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STUDIES ON TAPEWORM PHYSIOLOGY

IV. FURTHER OBSERVATIONS ON THE DEVELOPMENT OF *LIGULA INTESTINALIS IN VITRO*

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(With Plate 1 and Five Text-figures)

INTRODUCTION

The elaboration of an aseptic technique for the *in vitro* cultivation of cestodes has long been one of the most puzzling problems of parasitology. Within recent years, special attention has been paid to the pseudophyllidean cestodes, whose plerocercoids—being parasitic in the coelomic cavity of their hosts—offer special advantages for cultivation of this type.

The largest plerocercoid of this class of cestodes is that of *Ligula intestinalis* which parasitizes coarse fish, and whose adult form is found in fish-eating birds. It has been shown (Smyth, 1947*a*) that plerocercoids of *Ligula* removed aseptically from fish, develop into sexually mature tapeworms when cultured in peptone broth at 40° C. This development *in vitro* could not, however, be considered normal in that (*a*) the time required to reach sexual maturity *in vitro* (7 days) was nearly twice as long as that required for the larva maturing normally in the intestine of the bird host in the natural life cycle; (*b*) eggs produced *in vitro* were infertile. Similar experiments had previously been carried out by Joyeux & Baer (1938, 1942) who obtained only partial development, for although eggs were produced in some cases, the testes failed to undergo spermatogenesis.

The present paper covers a more detailed examination of some of the factors governing the development of *Ligula in vitro*. A brief note on some of these results has already been published (Smyth, 1948). Previous literature in this field has been reviewed in detail elsewhere (Smyth, 1947*c*).

MATERIAL AND METHODS

Plerocercoids of *Ligula* were obtained from infected roach taken from Thryberg Reservoir, Rotherham, England. The difficulties associated with the collection and transport of fish greatly restricted the supply of material, and it was not possible to use the same number of larvae in each experiment, or to carry out experiments on a scale as extensive as would be desirable. The range of culture media used was also restricted owing to the large quantities used in each experiment—some requiring as much as 0·5 l. per day.

The larvae were removed aseptically from fish by the technique already described (Smyth, 1947*a*) and cultured in 9 × 1 in. rimless culture tubes in an incubator at

40° C. For histological demonstration of fat, material was fixed in Baker's cadmium-formol solution, embedded in 25 % gelatine and frozen sections prepared and stained with Sudan Black. For demonstration of glycogen, material was fixed in hot picro-formol-alcohol, and embedded in Steedman's Ester wax according to the procedure recently developed (Smyth & Hopkins, 1949). Sections were stained in iodine or Best's carmine, using the excellent modifications of Bensley (1939).

ANATOMY OF PLEROCERCOID

The general anatomy of the plerocercoid has been described in detail (Joyeux & Baer, 1942; Smyth, 1947*a*), but the salient points will be summarized.

The larva is unsegmented but has a rugose appearance. Rudiments of the genitalia are present and appear in whole-mount preparations as thin band-like structures alternating irregularly on each side of the middle line. Detailed histological examination reveals that although the *anlagen* of the genitalia are present, they are in a poorly developed condition and consist of undifferentiated cells which stain very intensely with nuclear stains and which show little differentiation between nucleus and cytoplasm. Tubular genitalia (i.e. cirrus, vas deferens, vagina, and uterus) exist only as masses of cells, which although arranged to represent the outlines of these organs, show no trace of lumen formation. Genital pores are not present in the larva—the positions of the adult uterine, vaginal and cirrus openings being marked by thin solid cords of cells.

EXPERIMENTAL RESULTS

(1) *Nutrient media*

(*a*) *Meat-extract media.* The effect of using large and small quantities of medium and of renewing the medium frequently was determined with peptone-broth prepared from meat extract (Lab-Lemco) (Exps. 1–4, Table 1). The results were highly significant. Using only 20 c.c. per culture, larvae died within 1–4 days without ovipositing. One larva examined histologically after 3 days cultivation showed that spermatogenesis, oogenesis and vitelogenesis had commenced, but had only reached a very early stage of development. All cultures showed a very marked drop in pH, the maximum drop being from pH 8·0 to 5·0 in 3 days.

When 200 c.c. of medium was used per culture, the pH fell only from 8·0 to 6·0 in 9 days and three of the four larvae cultured oviposited on the 8th day (Exp. 2, Table 1). The cirrus was everted in some proglottides. Histological examination revealed that all organs had reached maturity. The testes contained up to four 'giant polyploid cells' per capsule.

In Exp. 3 (Table 1), in which 100 c.c. of medium was used (renewed 48-hourly) and two larvae were present in each tube, large quantities of eggs appeared within 4 days. Histological examination again revealed the presence of the 'giant polyploid cells' (Text-fig. 1) in the testes; as a rough approximation one was found per five capsules. The cirrus was everted in some proglottides (Pl. 1, fig. 2). The pH was not recorded.

Exp. 3 (Table 1) clearly indicated that frequent renewal of medium had a

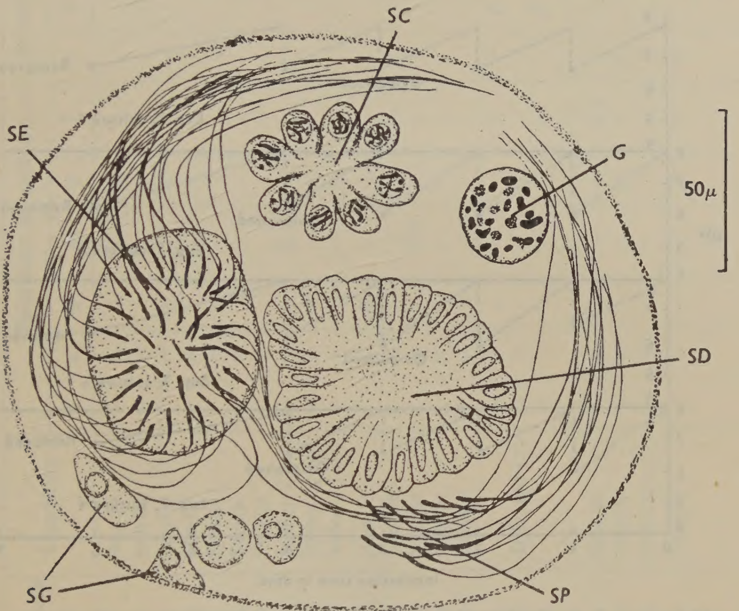
Table 1. *Results of cultivation of plerocercoids of* Ligula intestinalis *in nutrient media at* 40° C.

Exp. no.	Medium	Culture no.	Condition	Behaviour on each day of cultivation									Comments
				1	2	3	4	5	6	7	8	9	
1	1 % meat-extract + 0·5 % NacCl + 1 % peptone (20 c.c. one larva per tube)	1	Infected (0)	×	.	.	.	.	.	.	.	.	All larvae died without ovipositing
		2	Sterile	—	—	—	×	.	.	.	.	.	
		3	Sterile	—	—	*R*	.	.	.	.	.	.	
		4	Sterile	—	×	—	.	.	.	.	.	.	
		5	Sterile	—	—	×	.	.	.	.	.	.	
2	1 % meat-extract + 0·5 % NaCl + 1 % peptone (200 c.c. one larva per tube)	1	Infected (0)	—	—	—	—	—	—	—	*E*	×	All worms in sterile cultures oviposited. Eggs infertile
		2	Sterile	—	—	—	—	—	—	—	*E*	*R*	
		3	Sterile	—	—	—	—	—	—	—	*E*	*R*	
		4	Infected (0)	—	—	—	—	×	.	.	.	.	
3	1 % meat-extract + 0·5 % NaCl + 1 % peptone (100 c.c. two larvae per tube)	1 2	Infected (0)	—	—	—	*c*	—	×	.	.	.	Eight of the ten worms cultured oviposited. Eggs appeared after 96 hr. cultivation. Eggs infertile
		3 4	Infected (2)	—	*c*	—	*E* ×	.	.	.	.	.	
		5 6	Sterile	—	*c*	—	*Ec*	*R*	.	.	.	.	
		7 8	Infected (5)	—	*c*	—	*Ec*	—	*c*	*D*	.	.	
		9 10	Sterile	—	*c*	—	*Ec*	—	*R*	.	.	.	
4	1 % meat-extract + 0·5 % NaCl + 1 % peptone (50 c.c. one larva per tube)	1	Infected (0)	*R*	.	.	.	.	.	.	.	.	All sterile worms oviposited within 67 hr. Eggs infertile
		2	Sterile	*c*	*c*	*E*	*D*	.	.	.	.	.	
		3	Sterile	*c*	*c*	*E*	*R*	.	.	.	.	.	
		4	Infected (1)	*c*	*R*	.	.	.	.	.	.	.	
		5	Infected (3)	*c*	*c*	*E*	*D*	.	.	.	.	.	
5	1 % meat-extract + 0·5 % NaCl (50 c.c. one larva per tube)	1	Sterile	*c*	*c*	*Ec*	*R*	.	.	.	.	.	No. 4 was damaged. All others oviposited within 67–96 hr. Eggs infertile
		2	Sterile	*c*	*c*	*Ec*	*R*	.	.	.	.	.	
		3	Infected (0)	*c*	*c*	*Ec*	—	—	×	.	.	.	
		4	Sterile	*c*	*c*	—	—	—	×	.	.	.	
		5	Infected (1)	*c*	*c*	*Ec*	—	—	×	.	.	.	
		6	Infected (2)	*c*	*c*	*c*	*Ec*	*c*	—	*c*	*R*	.	
		7	Infected (2)	*c*	*c*	*E*	*c*	—	*c*	*c*	×	.	
6	1 % meat-extract + 0·5 % NaCl + 1 % peptone + 0·5 % glucose (50 c.c. one larva per tube)	1	Sterile	*c*	*c*	*c*	*c*	*c*	*c*	*c*	*R*	.	Only one worm oviposited. No. 1 had its scolex accidentally cut away. Eggs infertile
		2	Sterile	*c*	*c*	*c*	*c*	*c*	×	.	.	.	
		3	Sterile	*c*	*c*	*R*	.	.	.	.	.	.	
		4	Sterile	*c*	*c*	*c*	*ER*	.	.	.	.	.	
		5	Infected (4)	*c*	*c*	*c*	*c*	×	.	.	.	.	
		6	Infected (3)	*c*	*c*	—	*c*	×	.	.	.	.	
7	Pure horse serum (50 c.c. one larva per tube)	1	Sterile	*c*	*c*	*Ec*	*c*	—	*R*	.	.	.	All larvae oviposited within 70–100 hr. Eggs fertile
		2	Sterile	*c*	*c*	*c*	*c*	*E*	*R*	.	.	.	
		3	Sterile	*c*	*c*	*c*	*Ec*	—	*R*	.	.	.	
		4	Sterile	*c*	*c*	*c*	*Ec*	—	*R*	.	.	.	
		5	Sterile	*c*	*c*	*Ec*	*c*	—	*R*	.	.	.	
		6	Sterile	*c*	*c*	*c*	*Ec*	—	*R*	.	.	.	

Abbreviations: —, alive but medium unchanged; ×, dead; *E*, first appearance of eggs; *c*, medium changed; *D*, culture accidentally destroyed; *R*, worm removed and examined or fixed.

The number in brackets after 'infected' indicates the day on which the culture became infected. (0) indicates that culture was infected from the start of the experiment.

markedly beneficial effect and resulted in an increase in the rate of development as shown by the appearance of eggs within 4 days compared with 8 days in the earlier experiment. When the medium was renewed 24-hourly (Exp. 4, Table 1) eggs appeared within 67 hr., the pH never fell below 6·0, and larvae were considerably more active than in the previous experiment. Histological examination revealed a developmental picture similar to that in the previous experiment. It was obvious from this experiment that renewing the medium frequently was sufficient to induce development within a period (i.e. 67–72 hr.) equivalent to that required during the natural development in the bird gut. Conditions *in vitro* could now be said to be approaching those *in vivo*—as far as time of development could be used as a criterion. The exclusion of peptone from the medium (Exp. 5, Table 1) did not significantly affect the development.



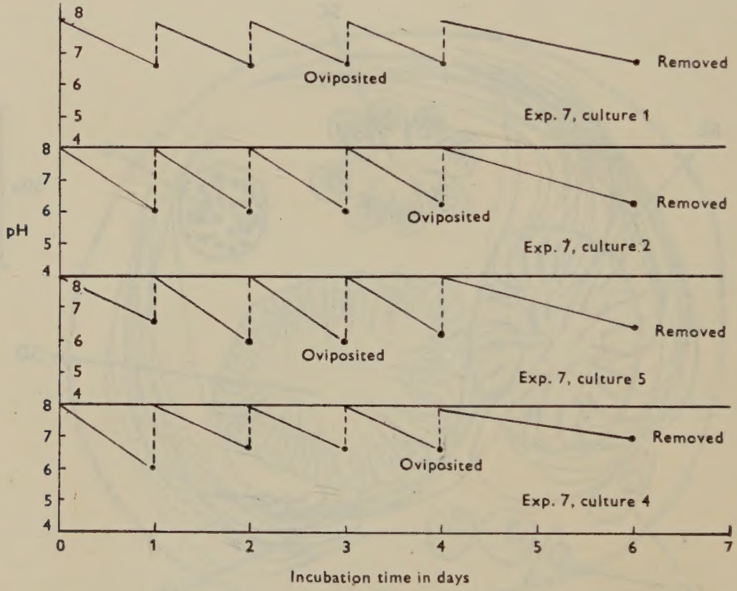
Text-fig. 1. A testis capsule of *Ligula* after cultivation for 72 hr. in nutrient medium. Slightly diagrammatic. *G*, giant polyploid cell; *SC*, spermatocyte morula; *SD*, early spermatid morula; *SE*, late spermatid morula; *SG*, spermatogonia; *SP*, spermatozoa.

The addition of glucose to the medium (Exp. 6, Table 1) appeared at first to be detrimental, because, of the four sterile cultures obtained, oviposition occurred in only one; but of these four cultures, no. 1 had its scolex accidentally removed. It would therefore be unwise to draw conclusions from such limited data.

(*b*) *Horse serum.* In 50 c.c. pure undiluted horse serum, renewed 24-hourly, all larvae oviposited within 70–100 hr., and the pH never fell below 6·0 (Text-fig. 2). In several cultures in this medium, the in-and-out copulating movement of the cirrus was plainly seen, and in one case the emission of the spermatozoa from the tip of the cirrus was observed. This emission is a very remarkable process to watch—the spermatozoa appearing as a small white cloud at the tip of the cirrus, rather like the puff of smoke from a gun. This experiment was abandoned after 6 days owing to lack of further supplies of serum, although all larvae were active and ovipositing intensively.

(2) *Balanced saline media*

In 50 c.c. unchanged $\frac{3}{4}$ Locke solution (Exp. 8, Table 2) all the larvae in sterile cultures died within 4 days without ovipositing; and the minimum pH recorded was 4·0. One larva in an infected culture (no. 8) oviposited on the 4th day, but died on the 5th. In the same medium renewed 24-hourly (Exp. 9, Table 2), one worm produced eggs on the 4th day. Histological examination of this worm revealed that development had taken place apparently in a similar manner to those cultured under similar conditions in nutrient media. The pH drop (Text-fig. 3) was considerably greater than in nutrient media.



Text-fig. 2. pH changes in four cultures of *Ligula* using horse serum as a medium renewed 24-hourly. Vertical dotted lines indicate renewal of medium. Serum supplies became exhausted after 4 days.

In Exp. 10 (Table 2), three larvae were cultured in 500 c.c. $\frac{3}{4}$ Locke solution. This culture was infected from the outset, and although one larva died, underwent rapid autolysis and went into solution on the 4th day, the remaining two remained active and both oviposited on the 8th day. The final pH was 7·0.

(3) *Buffered saline media*

In an attempt to neutralize acid produced during development, a few cultures were prepared with $\frac{3}{4}$ Locke solution with the addition of solid $CaCO_3$, or buffered with Na_2HPO_4—the medium being unchanged throughout.

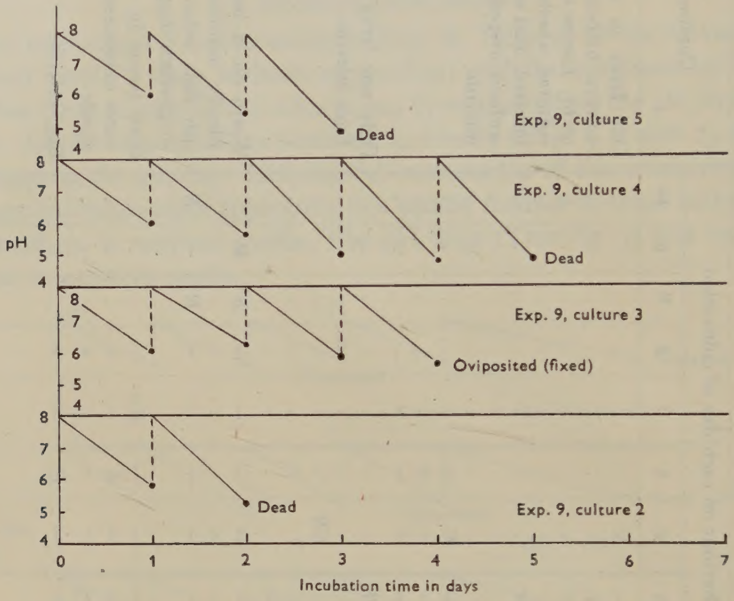
The presence of $CaCO_3$ (Exps. 11 and 12, Table 2) considerably reduced the rate of the pH drop—the pH never falling below 5·8 even after 7 days cultivation

Table 2. *Results of cultivation of plerocercoids of* Ligula intestinalis *in saline solutions at* 40° C.

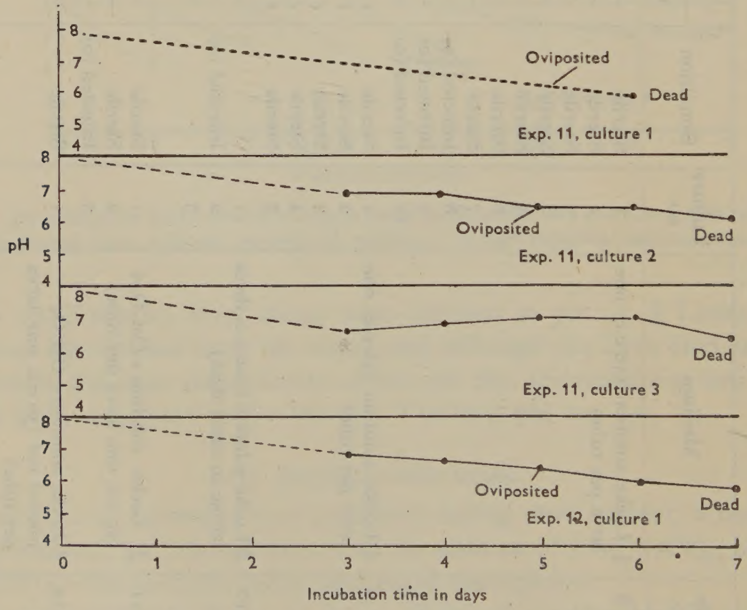
Abbreviations as Table 1.

Exp. no.	Medium	Culture no.	Condition	Behaviour on each day of cultivation 1	2	3	4	5	6	7	8	9	10	Comments
8	¾ Locke solution (50 c.c. one larva per tube)	1	Sterile	—	—	—	×	.	.	.	.	.	.	One larva in an infected culture oviposited within 96 hr. Larvae in remaining cultures, whether sterile or infected, died within 6 days without ovipositing. Eggs infertile
		2	Sterile	—	—	×	.	.	.	.	.	.	.	
		3	Sterile	—	—	×	.	.	.	.	.	.	.	
		4	Sterile	—	×	.	.	.	.	.	.	.	.	
		5	Sterile	—	—	×	.	.	.	.	.	.	.	
		6	Sterile	—	×	.	.	.	.	.	.	.	.	
		7	Sterile	—	×	.	.	.	.	.	.	.	.	
		8	Infected (o)	—	—	—	*E*	×	.	.	.	.	.	
		9	Infected (o)	—	—	—	—	×	.	.	.	.	.	
		10	Infected (o)	—	—	—	—	—	×	.	.	.	.	
9	¾ Locke solution (50 c.c. one larva per tube)	1	Sterile	*c*	*c*	*R*	.	.	.	.	.	.	.	Only one larva oviposited. Eggs infertile
		2	Sterile	*c*	×	.	.	.	.	.	.	.	.	
		3	Sterile	*c*	*c*	*c*	*ER*	.	.	.	.	.	.	
		4	Sterile	*c*	*c*	*c*	*c*	×	.	.	.	.	.	
		5	Sterile	*c*	*c*	×	.	.	.	.	.	.	.	
10	¾ Locke solution (500 c.c. three larvae in same flask)	1	Infected (o)	—	—	—	—	—	—	—	*E*	*R*	.	Worm no. 2 became completely autolysed by the 4th day other two both oviposited. Eggs infertile
		2		—	—	—	×	.	.	.	.	.	.	
		3		—	—	—	—	—	—	—	*E*	—	*R*	
11	¾ Locke solution + $CaCO_3$ + (50 c.c. one larva per tube)	1	Sterile	—	—	—	—	—	*E* ×	.	.	.	.	Both larvae in sterile cultures oviposited. Eggs infertile
		2	Sterile	—	—	—	—	*E*	—	×	.	.	.	
		3	Infected (o)	—	—	—	—	—	—	×	.	.	.	
12	¾ Locke solution + $CaCO_3$ phenol red (50 c.c. one larva per tube)	1	Sterile	—	—	—	—	*E*	—	×	.	.	.	Eggs infertile
13	¾ Locke solution + Na_2HPO_4 (50 c.c. one larva per tube)	1	Sterile	—	—	—	×	.	.	.	.	.	.	Both larvae died without ovipositing
		2	Sterile	—	—	—	—	×	.	.	.	.	.	

(Text-fig. 4). In Exp. 12, 0·1 g. of solid phenol red was added to the culture in addition to the $CaCO_3$. At the beginning of cultivation the colour was bright red (pH 8·0). After 16 hr. the lower half of the tube was yellow, while the upper half



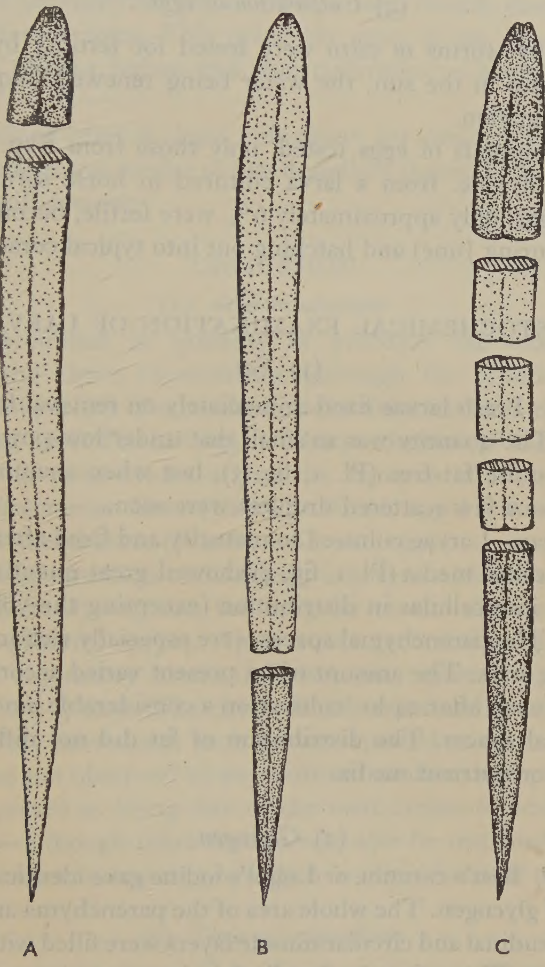
Text-fig. 3. pH changes in four cultures of *Ligula* using ⅗ Locke solution as a medium; renewed 24-hourly. Vertical dotted lines indicate change of medium.



Text-fig. 4. pH changes in four cultures of *Ligula* using ⅗ Locke solution $+CaCO_3$ as a medium. Medium unchanged throughout.

retained almost its original colour. This demonstrated the production of acid, and also showed that activity during the early stages of cultivation must have been slight —otherwise considerable mixing would have occurred. The culture was shaken at intervals during the subsequent cultivation period and by the 3rd day had become orange-red; and on the 5th day it had become yellow.

Of the two larvae cultured in saline-phosphate, one died on the 4th day and one on the 5th day without ovipositing. The final pH were 5·6 and 5·4 respectively.



Text-fig. 5. Plerocercoids of *Ligula* showing cuts made before cultivation. Lightly shaded parts were cultured; heavily shaded parts were discarded.

(4) *Development of plerocercoid fragments*

The following fragments were cultured in peptone-broth renewed 24-hourly: (*a*) larva with scolex removed (Text-fig. 5 A); (*b*) larva with posterior region removed (Text-fig. 5 B); (*c*) three pieces about 1 cm. long cut from the middle region of a large plerocercoid (Text-fig. 5 C). (*a*) and (*b*) showed less activity than normal plerocercoids, but both produced eggs on the 4th day. In (*c*), each of the fragments showed very little undulant activity and when removed from the medium on the 4th day, eggs were absent from the medium but present in the uteri of the teased fragments. Histological examination revealed that normal development had taken place in all cases. There was no evidence of mitosis at any of the cut ends, which suggested that tissue regeneration had not taken place.

(5) *Cultivation of eggs*

Eggs produced by worms *in vitro* were tested for fertility by being placed in shallow watch-glasses in the sun, the water being renewed frequently to combat microfloral contamination.

Of the many thousands of eggs tested, only those from Exp. 7, Table 1 (only culture no. 1 tested)—i.e. from a larva cultured in horse serum—proved to be fertile. Of these eggs, only approximately 6% were fertile, becoming embryonated within 6–7 days (during June) and hatching out into typical coracidia in 9–10 days.

HISTOCHEMICAL EXAMINATION OF LARVAE

(1) *Fat*

(*a*) *Fresh larvae.* Fresh larvae fixed immediately on removal from fish contained only traces of fat. The quantity was so small that under low-power observation the sections appeared quite fat-free (Pl. 1, fig. 3), but when examined under an oil-immersion objective a few scattered droplets were seen.

(*b*) *Cultured larvae.* Larvae cultured to maturity and fixed after 7–9 days cultivation in nutrient or saline media (Pl. 1, fig. 4) showed great quantities of fat present, which was mostly intercellular in distribution (excepting the yolk glands where it was intracellular). The parenchymal spaces were especially rich in fat in the form of droplets of varying sizes. The amount of fat present varied according to the period of cultivation, but even after 24 hr. cultivation a considerable amount had appeared in the parenchymal spaces. The distribution of fat did not differ between larvae cultured in saline or nutrient media.

(2) *Glycogen*

(*a*) *Fresh larvae.* Best's carmine or Lugol's iodine gave identical results regarding the distribution of glycogen. The whole area of the parenchyma and the intercellular spaces in the longitudinal and circular muscle layers were filled with great amorphous masses of glycogen (Pl. 1, fig. 1). Small globules were also present in the small lumina of the longitudinal muscles as viewed in transverse section. The subcuticular spaces were also filled with glycogen.

(*b*) *Cultured larvae.* Larvae cultured in meat-extract media were examined at intervals during cultivation. After 8 days—i.e. when egg production had been continuing for 5 days—sections revealed that a considerable quantity of glycogen was still present. The glycogen in the parenchyma—though still present in quantity—was more in the form of large droplets rather than in the great amorphous masses found in the fresh larvae. The subcuticular region appeared as rich in glycogen as in the fresh larvae.

It is difficult to assess accurately the amount present by comparison with sections of fresh larvae because the latter are wide in transverse section, have a large parenchymal area and small and poorly developed genitalia; whereas the mature worm has the genitalia in a much more highly developed condition which—in combination with the relaxation (and subsequent narrowing) of the worm—greatly reduces the parenchymal area. Comparison would, therefore, have to be made between different areas. This being the case, it can be concluded merely that glycogen was present before and after cultivation; a quantitative comparison is not possible.

In worms examined after 6 days' cultivation (or less) in saline solutions the distribution of glycogen was similar to that described above. Considerable quantities of glycogen were still present.

DISCUSSION

(1) *Acid production*

That acids are produced in quantity is probably the most important fact ascertained from the above experiments, although the production of acids by cestodes during brief periods of non-aseptic cultivation has been reported previously by Brand (1933) and Alt & Tischer (1931). This production of acid is of considerable interest, for there is some evidence that the anaemia often associated with the presence of *Diphyllobothrium latum* (Castle & Minot, 1936) is due to the toxic action of acid products of metabolism (Wardle & Green, 1941).

In vitro, the acid by-products undoubtedly interfere with normal development—a fact which satisfactorily explains the irregular results obtained in the previous paper in this series (Smyth, 1947*a*). Apart from a general slowing down of development, the general copulatory mechanism was also upset. Most larvae cultured everted the cirrus in 3–5 days. The cirrus usually remained everted and the typical in-and-out copulatory movement, such as was reported to occur in *Schistocephalus* (Smyth, 1946), was not observed in any nutrient medium except serum. This can probably be interpreted as being due to the toxic action of acid which is largely buffered in serum—although other factors may also be responsible. The pH drop in serum certainly was considerably less than in other nutrient media.

(2) *Time of oviposition*

As a result of the slowing down of development due to acid production, the time required to become sexually mature was largely dependent on the frequency of change of medium, and it was necessary to use 50 c.c. of medium renewed 24-hourly in order to obtain oviposition within 3 days or less. Donnadieu (1877) reported that eggs of *Ligula* appeared in duck faeces within 2 days—a fact quoted by Cooper (1918), and which the present writer in a previous communication (1947*a*) assumed to be correct. If this figure is, in fact, correct, the period of 67 hr. —the lowest obtained in the present *in vitro* experiments—is still too long, and the development therefore cannot be considered normal. Detailed reference to Donnadieu's paper, however, showed that he could not differentiate between *Ligula* and *Schistocephalus* (which is remarkable considering the external differences) —a fact which leaves little doubt that the worm which produced eggs within 36 hr. in his experiments was *Schistocephalus* and not *Ligula*. All other evidence from feeding experiments on birds indicates that eggs never appear in the faeces in less than 3 days (Joyeux & Baer, 1942; Rosen, 1918). It is concluded therefore that, in suitable media changed 24-hourly, the production of eggs within 3 days indicates normal development.

(3) *Histological changes*

Thanks to the generosity of the French helminthologists, Prof. Joyeux and Prof. Baer, and Dr Baylis of the British Museum, I have had an opportunity of examining sections and strobila of two normal adult *Ligula* from birds. Although the material had been rather poorly fixed, it was possible to work out the general histological features. It was found that, with the exception of the 'giant cells' in the testes, there was no significant difference between these worms and the specimens matured artificially *in vitro*. Structures similar to these 'giant cells' (Text-fig. 1) were seen in one worm but not in the other. Although these cells may well represent 'nurse' cells of some kind, in the present experiments it has been shown that they appear to be more frequent in worms cultured in unchanged media (i.e. abnormal conditions)—although it is difficult to make a quantitative estimation. It is tentatively suggested that the development of these cells is induced by the presence of toxic acid by-products in cultures. Similar cells have been reported previously in *Schistocephalus* artificially matured *in vitro*. If the structures observed in the specimens from birds do, in fact, represent 'giant cells', their presence could be accounted for by supposing that in the case of a heavy infestation, the acid by-products produced by the worms cannot be absorbed sufficiently rapidly by the gut wall. It is to be expected therefore that there will be considerable variation even between so-called 'normal' worms matured in the gut environment.

(4) *Fertilization*

Since fertile eggs were obtained from a *single* plerocercoid cultured in horse serum, it can definitely be concluded that *Ligula* is capable of self-fertilization, and that copulation between different worms is not a pre-requisite of fertility. Fertilization *in vitro* must take place merely by the migration of ejected spermatozoa into the vaginal pore of the same or adjacent proglottides. Obviously, in a liquid medium, the chance of spermatozoa reaching the vagina is small as compared with a worm confined within the narrow host gut where the concentration of spermatozoa would be correspondingly higher. This probably accounts for the low proportion of fertile eggs obtained.

It is difficult to comment adequately on the fact that fertile eggs were obtained only from a worm cultured in serum and not in other media. It may be that the physical or chemical properties of serum are more suited to the worm (or to the ejected spermatozoa), but it is questionable whether such a conclusion is justifiable on such slender evidence. It is open to criticism on technical grounds in that the percentage fertility in serum was so low that fertile eggs may possibly have been obtained from larvae cultured in other media, but that the egg-testing technique may not have been adequate for the detection of lower degrees of fertility. This problem must await further and more detailed investigation.

(5) *Metabolism*

The present results throw little light on metabolism. The marked production of cytoplasmic fat is strong evidence in support of the hypothesis that fat is a by-product of metabolism, a result which agrees with that obtained with *Diphyllobothrium* (Smyth, 1947*b*). It cannot be concluded from available evidence that this cytoplasmic fat is formed from glycogen, for it has been shown that even under conditions of starvation, a considerable quantity of glycogen was still present after 6 days' cultivation. This latter result is surprising, for it has been shown by Reid (1942) and Markov (1939) that certain cestodes undergo rapid loss of glycogen under conditions of starvation. Since *Ligula* larvae can undergo development in saline solutions alone, there seems little doubt that the carbohydrate reserves are sufficient to enable them to live for considerable periods at a high metabolic rate (i.e. at 40° C.) under conditions of starvation. There is thus no need to provide a source of carbohydrate in the medium for larvae to undergo development. The effect of adding glucose to the medium was not beneficial, and possibly it was deterimental, although the evidence was not entirely convincing. Evidence from previous work (Smyth, 1946, 1947*b*) has also suggested that glucose in the medium may be unfavourable to cultural conditions.

(6) *General comments*

The question of the conditions under which normal development of *Ligula* will take place *in vitro* is extremely complicated. There seems little doubt that serum is the best of the nutrient media so far used, but as already indicated this is probably on account of its high buffering properties. Probably if the acid by-products can be disposed of sufficiently rapidly, increase in temperature to 40° C. alone is sufficient to induce development and no nutritional requirements need be fulfilled. In this respect the behaviour of larvae in saline solutions is particularly interesting, but on the limited data available the position is difficult to elucidate. The fact that three out of the five larvae cultured in saline solutions (renewed 24-hourly) died without ovipositing, suggests that development in this medium was abnormal. On the other hand, one larva oviposited in 87 hr. and showed apparent normal histological development. Again in saline solution + $CaCO_3$—in which the pH fall was slow—all three larvae in sterile cultures oviposited in unchanged media although the earliest oviposition time was 5 days. Therefore, although development is possible in non-nutrient media, the precise conditions under which such development can occur are not known.

The fact that certain bacterial infections have no detrimental effect on development is in contrast with the view usually held, i.e. that asepsis is essential for successful *in vitro* cultivation (Wardle, 1937; Smyth, 1947*c*). Indeed, in one case

(Exp. 8, no. 8) the presence of bacteria apparently allowed a larva to undergo development in 4 days in 50 c.c. of *unchanged* saline medium. This remarkable result is possibly due to alkaline bacterial by-products neutralizing the acid cestode by-products, for the pH of this particular culture was 5·5 on the 4th day compared with 4·8 for a worm of similar size in sterile culture. The majority of infected cultures die rapidly and there is little doubt that most bacteria produce substances which are toxic to *Ligula*. The relationship between *Ligula* and the presence of bacteria in cultures is worthy of further investigation; and it would be interesting to determine whether cestodes (or indeed any parasitic helminth) is capable of producing substances with bacteriostatic properties.

The fact that larvae without scoleces, or small fragments of larvae containing genitalia, can undergo maturation and oviposit, opens up interesting possibilities for further work in the field of experimental embryology—for plerocercoid larvae can be considered to consist of partly differentiated embryonic tissue. This ability to produce eggs even from fragments would be advantageous to a larva, for during its passage down the alimentary canal of a bird, it doubtless receives rough treatment in the stone-filled gizzard and must frequently be broken up or mutilated in some way. These results, however, are in contrast with those obtained by previous workers who have fed mutilated larvae to birds. Donnadieu (1877) found that only fragments of *Ligula* (or *Schistocephalus*?) bearing scoleces became mature when fed to ducks. Joyeux & Baer (1942) fed larvae, from which the scoleces had been removed, to a duck and found that even after 10 days no eggs or fragments were passed out in the faeces.

Although these experiments may be considered relatively successful in that they represent the first time fertile eggs of a cestode—or indeed any parasitic helminth—have been obtained *in vitro*, the scarcity of material has somewhat limited their scope. A detailed biochemical analysis of larvae before and after cultivation, and a similar analysis of the medium—especially the acid by-products—could throw much light on the metabolism of cestodes. Further investigation of the effect of varying certain specific controlling factors—pH, temperature, osmotic pressure, presence of vitamins, hormones or bacteria in the medium—to name the most important, is also indicated. The production of viable eggs opens up the way to possible *in vitro* cultivation of coracidia and procercoid larvae—the final aim being to complete, if possible, the entire life cycle of this cestode *in vitro*.

SUMMARY

1. Plerocercoid larvae of the pseudophyllidean cestode *Ligula intestinalis* from the body cavity of roach, were cultured *in vitro* at 40° C. in a variety of saline and nutrient media. About 65% of such cultures were aseptic.

2. During cultivation, larvae produced acid by-products (unidentified) and the pH fell rapidly.

3. The presence of these acid by-products slowed down development, or, if present in sufficient quantity, caused death.

4. In order to obtain development in nutrient media in a period (3 days) comparable to that required in a bird (the normal host) it was necessary to renew the medium 24-hourly.

5. 6% of the eggs produced from a worm cultured in horse serum were fertile. Fertile eggs were never obtained from larvae cultured in any other media.

6. Certain bacterial infections had no apparent detrimental effect on development, but others were toxic.

7. Some larvae underwent development in non-nutrient medium ($\frac{3}{4}$ strength Locke's solution). The exact conditions under which this occurred was not determined.

8. Fragments (3 cm. long), of larvae or larvae with either scolex or posterior half removed, underwent development to the stage of oviposition in nutrient media.

9. Histochemical examination revealed that the plerocercoid larvae were almost fat-free. During cultivation, very large quantities of cytoplasmic fat were produced—the quantity being proportional to the duration of cultivation. Fat was produced even under starvation conditions (i.e. during cultivation in saline) and can be considered a metabolic by-product.

10. The fresh plerocercoid contained great quantities of glycogen in the parenchyma and muscle regions. After cultivation in nutrient or saline media, considerable quantities were still present.

My best thanks are due to the following for help with collection and transport of infected fish: Prof. E. A. Spaul and Mr T. Kerr of the Zoology Department, Leeds University; Mr W. H. Price, Doncaster Borough Surveyor; Mr G. Witham, Secretary of the Doncaster Angling Association. The major part of this work was carried out in the Zoology Department, Leeds University, England.

REFERENCES

Alt, H. L. & Tischer, O. A. (1931). *Proc. Soc. Exp. Biol., N.Y.*, **29**, 222.
Bensley, C. (1939). *Stain. Tech.* **14**, 47.
Brand, Th. v. (1933). *Z. vergl. Physiol.* **18**, 562.
Castle, W. B. & Minot, G. R. (1936). *Pathological, Physiological and Clinical Description of Anaemias.* Oxford.
Cooper, A. R. (1918). *Illinois Biol. Monogr.* **4**, 16.
Donnadieu, A. L. (1877). *C.R. Soc. Biol., Paris*, **100**, 619.
Joyeux, Ch. & Baer, J. G. (1938). *C.R. Soc. Biol., Paris*, **129**, 314.
Joyeux, Ch. & Baer, J. G. (1942). *Bull. Mus. Hist. nat. Marseilles*, **2**, 1.
Markov, G. S. (1939). *C.R. Acad. Sci. U.R.S.S.* **25**, 93.
Reid, W. M. (1942). *J. Parasit.* **28**, 319.
Rosen, F. (1918). *Bull. Soc. Neuchatel. Sci. nat.* **44**, 259.
Smyth, J. D. (1946). *J. Exp. Biol.* **23**, 47.
Smyth, J. D. (1947*a*). *Parasitology*, **38**, 173.
Smyth, J. D. (1947*b*). *J. Exp. Biol.* **24**, 374.
Smyth, J. D. (1947*c*). *Biol. Rev.* **22**, 214.
Smyth, J. D. (1948). *Nature, Lond.*, **161**, 138.
Smyth, J. D. & Hopkins, C. A. (1949). *Quart. J. Micr. Sci.* (in the Press).
Wardle, R. A. (1937). *Manitoba Essays.* Toronto.
Wardle, R. A. & Green, N. K. (1941). *Trans. Roy. Soc. Canad.* (v), **35**, 85.

EXPLANATION OF PLATE 1

Fig. 1. Transverse section of an uncultured plerocercoid showing quantitites of glycogen localized mainly in the parenchyma and muscular regions. Picro-formol-alcohol. Best's carmine. × 30.

Fig. 2. Transverse section of mature worm showing cirrus everted and eggs in uterus. Exp. 3, culture 9. Cultured in meat-extract-peptone for 6 days; medium renewed 48-hourly; two larvae per 100 c.c. of medium. Bouin. Heidenhain's haematoxylin. × 36.

Fig. 3. Part of a transverse section of a fresh uncultured plerocercoid. Fat almost completely absent. Fixation and staining as in Fig. 4.

Fig. 4. Part of a transverse section showing numerous fat globules in parenchymal and muscular regions. Exp. 10, no. 1. Cultured ¾ Locke solution for 9 days; three larvae per 500 c.c. Formol-cadmium-saline. Gelatine embedded. Sudan black. × 50.

Abbreviations: *C*, cirrus; *E*, eggs; *Ex*, excretory canal; *M*, muscle region; *P*, parenchyma; *R*, receptaculum seminis; *T*, testes; *Y*, yolk glands.

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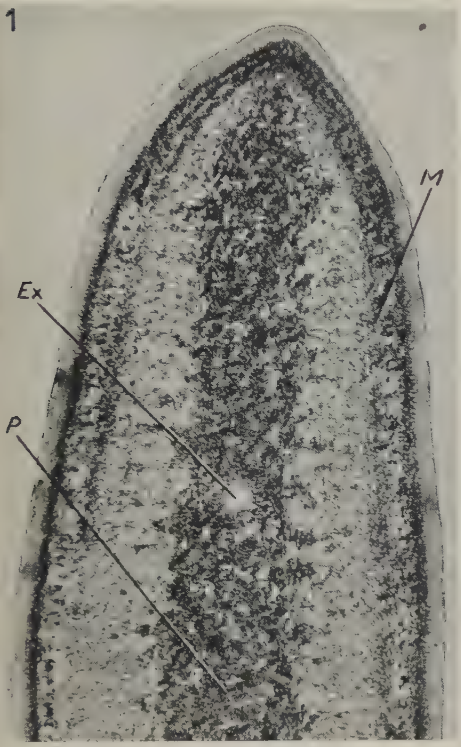

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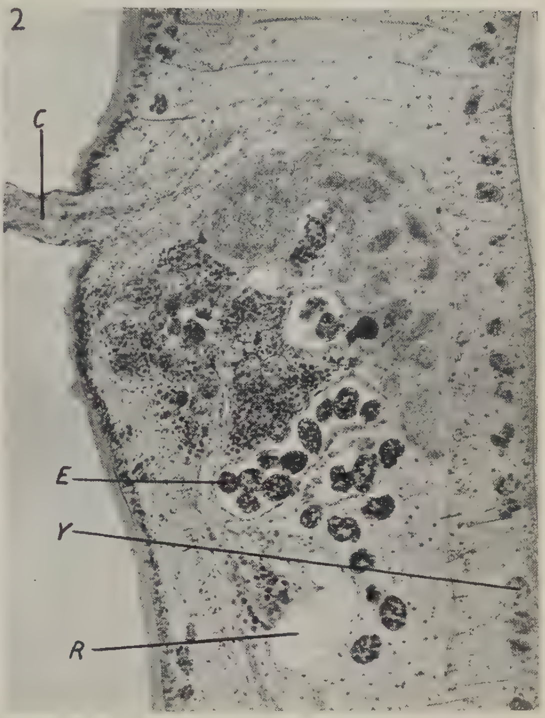

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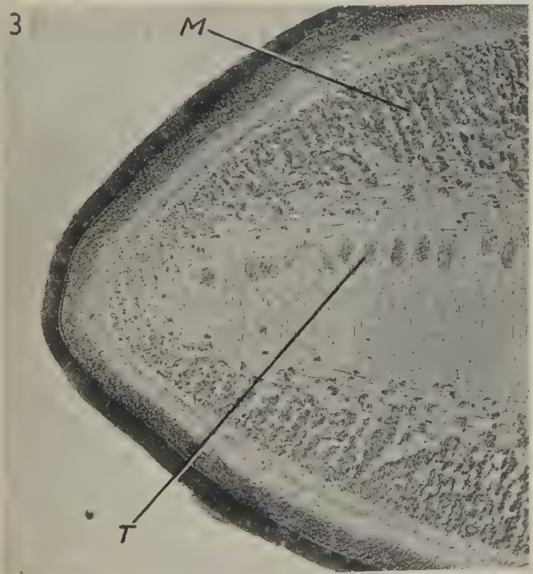

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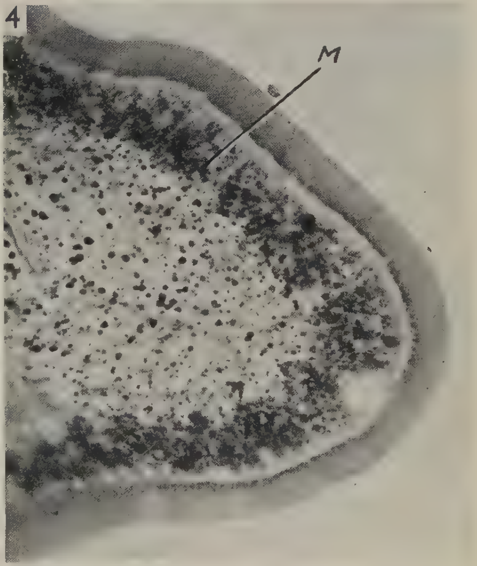

SMYTH—STUDIES ON TAPEWORM PHYSIOLOGY

A SIMPLIFIED ULTRA-MICRO KJELDAHL METHOD FOR THE ESTIMATION OF PROTEIN AND TOTAL NITROGEN IN FLUID SAMPLES OF LESS THAN 1·0μl.

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(With Three Text-figures)

INTRODUCTION

In adding to the already swollen list of micro-modifications of the Kjeldahl method our object has been the simplification of apparatus and procedure without loss of sensitivity. The method which we have evolved requires no apparatus which cannot easily be made from ordinary materials without special facilities, and we have introduced a new feature in the separate estimation of protein nitrogen. The quantities of nitrogen which can be estimated range from 1·0 to 10·0 μg. contained in 0·5–1·0 μl. with a standard deviation of 0·02–0·04 μg. N. We have thus been able to make duplicate determinations of protein and total nitrogen, as well as of chloride by Wigglesworth's micro-method and of osmotic pressure by Baldes' thermoelectric technique, using only a fraction of the blood of an insect larva (such as *Sialis lutaria* or *Aeschna cyanea*) from which further samples can be taken later.

Most of the previous methods of adapting procedure to this scale have been reviewed by Conway (1947). Their main features are set out in Table 1. Especial difficulties are encountered in: (1) accurate delivery of the sample into the digestion vessel; (2) transference of the digest to the diffusion vessel; (3) adjustment of volumes and concentrations of reactants and design of diffusion vessel to ensure complete absorption of the ammonia in a reasonable time with no danger of direct contact between alkalinized digest and absorbing standard acid; (4) design of diffusion vessel so that the acid can afterwards be titrated *in situ*; (5) evolving a sufficiently sensitive titration; (6) avoidance of contamination by extraneous nitrogen.

Of the methods listed in Table 1 we have the practical experience of Needham & Boell (1939) and Brüel, Holter & Linderstrøm-Lang (1946). That of Needham & Boell entails specially constructed combined digestion and diffusion vessels and a rocking device. The digestion, which involves the adjustment of micro-burners, we have found difficult and tedious, and not suitable for the simultaneous treatment of more than a few samples. Owing to the small surface of acid exposed, the absorption time is as much as 12 hr. at 37° C. Boell's method (Table 1*e*), though simple, does not attain the required sensitivity. Tompkins & Kirk (1942) used a digestion-diffusion vessel somewhat similar to that of Needham & Boell (1939), but reduced the diffusion time and increased the accuracy by exposing the standard acid in a spoon-shaped cup attached to the stopper. The method of Brüel *et al.* (1946) is the simplest as regards apparatus and we have adopted their technique for the digestion. But their diffusion procedure depends upon the subsequent method of titration, which we have simplified, and in general we have found the method tedious and requiring constant practice.

Table 1

Authors	Quantity of nitrogen (μg.) in sample	Standard deviation (μg. N)	Diffusion technique	Diffusion time (hr.)	Titration
(*a*) Bentley & Kirk (1937)	1·0–10·0	0·05–0·10	In digestion vessel	2 (50° C.)	Rehberg type burette
(*b*) Borsook & Dubnoff (1939)	5·0–10·0	0·05	Transfer from digestion tube to Conway type diffusion vessel	1–2 (room temp.)	Electrometric
(*c*) Needham & Boell (1939)	1·0–10·0	0·3	In digestion vessel	18 (37° C.)	Rehberg burette, magnetic stirring
(*d*) Tompkins & Kirk (1942)	0·5–10·0	0·02	In digestion vessel	5 (room temp.)	Rehberg burette
(*e*) Boell (1945)*	1·0–50·0	>0·5	Digest diluted and aliquot transferred to diffusion vessel	1½ (room temp.)	Rehberg burette
(*f*) Brüel *et al* (1946)*	0·1–1·0	0·005	Transfer from digestion tube to waxed diffusion tube	1–2 (40° C.)	Rehberg type burette, magnetic stirring
(*g*) This paper*	1·0–10·0	0·02–0·04	Transfer from digestion tube to hanging drop diffusion vessel	7 (room temp.)	Simple capillary burette, air-jet stirring
	0·1–1·0	0·006	(Diffusion only)		

* Not reviewed by Conway (1947).

The technique described in this paper involves the following features not entailed in the methods listed in Table 1: (1) waxed capillary pipettes modified from the Wigglesworth (1938) type for quantitative delivery of the sample and of the standard acid; (2) separation of the protein sample; (3) a type of diffusion vessel designed to house a hanging drop of standard acid on a waxed slide; (4) final titration with an easily constructed and simply operated microburette,* the drop on the waxed surface being stirred with an air jet as in Wigglesworth's micro-chloride method (1938).

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* Attempts to make a waxed capillary burette of the Wigglesworth type failed. It was found impossible to produce a stable wax film in a capillary fine enough to give the required sensitivity, which is considerably finer than that used for the chloride titration. Moreover, it is unlikely that a wax film would remain intact for long in presence of N/10 alkali.

General points on procedure and apparatus

In the dirt-laden atmosphere of Newcastle it was found necessary to keep all apparatus and reagents under dust covers and to perform all operations under a canopy which excludes falling particles.

The distilled water was prepared free of ammonia by redistillation from glass after addition of sulphuric acid and potassium permanganate. Blank determinations showed that these precautions were adequate.

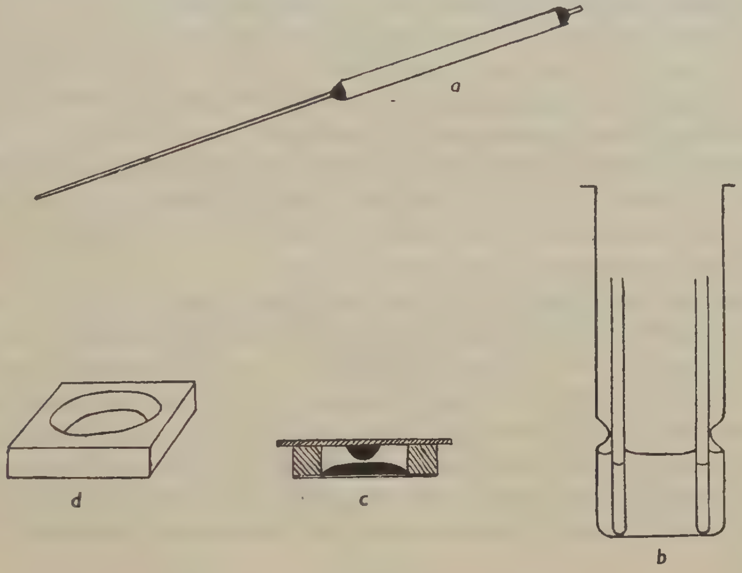

Fig. 1.

The waxed sample pipette is modified from the Wigglesworth type in that the capillary protrudes some 5 cm. from the end of the supporting tube in order that the bottom of the narrow digestion tube can be reached (Fig. 1*a*). The sample (0·7–0·8 μl.) extends about 3 cm. from the tip, the upper end of the sample being marked by a fine spot of black paint. These pipettes have the advantage that the whole of the section holding the sample, not merely the tip, can be rewaxed by immersion and blowing under a wax bath. We have even succeeded in removing the wax film by drawing up xylol several times to just beyond the mark, followed by acetone, concentrated nitric acid to remove protein particles, acetone, xylol and finally rewaxing. In this way a pipette can be renovated after the wax film has broken down or particles of organic matter have become attached to it which is not possible with the original model. These modified pipettes are not in practice more fragile than the original and the renovation has so far made no detectable difference to capacity as determined by calibration for the micro-chloride method, for which we also use them. The drop of standard acid is delivered from a waxed pipette of the normal Wigglesworth type holding approximately 7 μl. The wax used in these operations is the B.D.H. 'so-called' paraffin wax (clearing point 65/71° C.).

Other pipettes are simple drawn-out tubes of suitable dimensions and not waxed. All are operated by rubber suction tubes with glass mouth pieces packed with filter-paper.

Digestion

The pyrex digestion tubes are of dimensions 55 × 2 mm. (Fig. 1 *b*).

For total nitrogen the sample is placed at the bottom of the tube followed by one washing of distilled water. For protein nitrogen a column about 4 mm. long of 20% trichloracetic acid is placed about half-way down the tube by means of a fine unwaxed pipette and the sample is added to this with one washing. After centrifuging to the bottom the supernatant acid is sucked from the precipitate and the tube is filled with 5% trichloracetic acid with careful washing down the sides of the tube. After further centrifuging and removal of acid this washing is repeated.

The procedure is now common to both samples.

A satisfactory digestion mixture was found in 50% sulphuric acid saturated with K_2SO_4 and containing 2% $CuSO_4$ and 0·6% SeO_2. About 12 μl. are placed as a column at the top of each tube and shaken to the bottom. The tubes are then left overnight in an oven at 105° C. to remove water.

The digestion is completed by the method of Brüel *et al.* (1946), the tubes being heated at 280–300° C. for 5 hr. in a bath of sulphuric acid saturated with K_2SO_4. The bath is a pyrex tube of dimensions about 6 × 2 cm. with a constriction as shown in Fig. 1 *b*, and is embedded to a depth of about 1 cm. in a heated metal block. By this method there is no spluttering and the operation requires little attention as it is comparatively easy to adjust a bunsen under the block to maintain the temperature within this range. Several baths, each containing 6–12 tubes, can be held in one block, and a large number of digestions can thus be done simultaneously and with little trouble.

Diffusion

The diffusion chambers, 13 mm. internal diameter and 5 mm. deep (Fig. 1 *c*, *d*) are cut from a brass plate, the bottom being of thin sheet-brass and soldered on. They could be made of other materials, but since the heat of neutralization and dilution might melt the wax coat there is possibly some advantage in using a good heat conductor. We have not experienced any trouble of this sort. The diffusion chambers are coated by total immersion in molten wax (B.D.H. clearing point 65/71° C.). Glass micro-slides are similarly coated with pure paraffin wax (m.p. 48° C.). The rim of each chamber is then greased with Conway's fixative (50 g. paraffin mp. 48° C. mixed by heating with 80 ml. of liquid paraffin).

A saturated solution of potassium metaborate (50–70 μl.) is placed in each chamber to one side, and on the other the contents of a digestion tube with about 80 μl. of distilled water used as washings. The transfer of the digest is made with a fine pipette followed by careful washing down the sides of the tube with three lots of distilled water. The technique is in fact that of Brüel *et al.* (1946), except that the transfer is direct to the chamber and not via a wax block.

About 7 μl. of 0·1 N-HCl delivered quantitatively from a waxed pipette is placed as a drop in the centre of each waxed cover-slide. This volume is suitable for the estimation of 3–7 μg. nitrogen. The indicator for the final titration is incorporated in this acid as well as in the standard alkali in the burette. It is made up according to Conway (1947, p. 55)—1·3 ml. 0·1 % methyl red and 0·66 ml. 0·1 % bromocresol green per 100 ml. of standard solution.

Each cover is then inverted and fixed in position immediately after shaking the chamber sufficiently to bring the digest and metaborate together (Fig. 1 *c*). We have found that no significant ammonia is lost in this way, whereas the hanging drop of acid is apt to be displaced if the shaking is done after the cover is in position.

The time allowed for diffusion is 7 hr. at room temperature (see section on diffusion time).

Titration

The burette is made from the lower end of a broken thermometer tube about 10 cm. long. The tube which we have in use holds about 7 μl. for 140 divisions on the thermometer scale and tests have shown that the bore is sufficiently uniform to enable us to use these divisions. In fact a good quality thermometer of suitable bore is required. The previous bulb end is fused to another tube and drawn out to a capillary bent at right angles near its origin (Fig. 2).

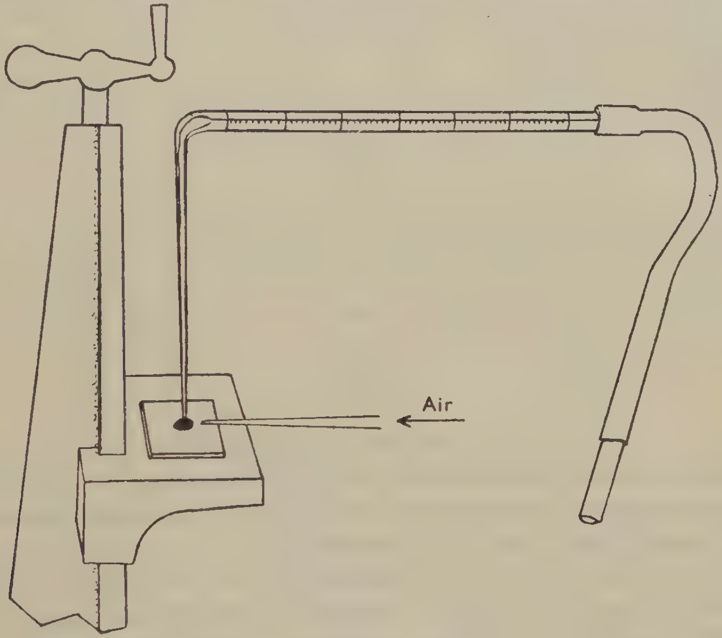

Fig. 2.

The calibrated tube is fixed horizontally with the drawn-out capillary directed vertically downwards. The length of the capillary and the bore at the tip are so adjusted that, when the burette is filled with a mouth-suction tube, there is no discharge until the tip touches the drop of acid on the waxed slide raised from below on an adjustable screw stand (Fig. 2). An air jet is fixed relative to the tip of the burette so as to stir the drop when raised into position. The horizontal setting of the upper calibrated portion of the burette ensures that there is no change in height of the liquid column and thus the rate of delivery is uniform, which is essential for accurate quantitative delivery.

The burette is filled with 0·1 N-NaOH with added indicator (see above) from a drop placed on a freshly waxed slide. The end-point (red to green) is sharp when seen against a white and well-illuminated background. The titration is controlled by raising and lowering the screw-stand.

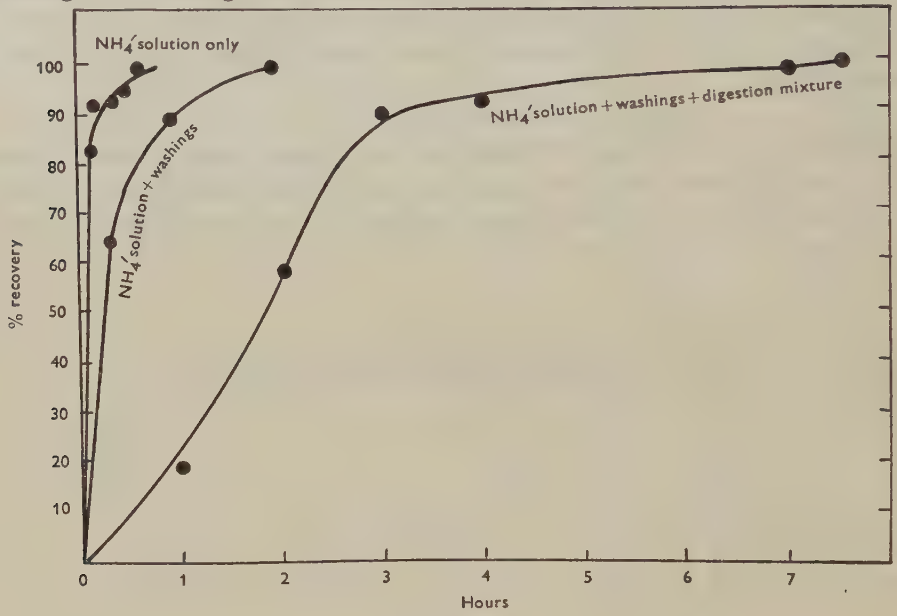

Fig. 3.

Calibration and calculation of results

No solutions are accurately and independently standardized except the acid used as ammonia absorbent. From this the 0·1 N-NaOH is repeatedly standardized by titration in terms of scale units on the burette.

It is also unnecessary to determine accurately the volume of any of the pipettes. It is required only to know the ratio-volume of standard acid pipette/volume of blood-sample pipette (A/B). This can be found by filling each with standard acid and titrating against alkali in the burette.

If a_1 scale units of alkali are required to neutralize one pipette load of standard acid, and a_2 are needed after absorption of the ammonia, then the concentration of nitrogen in the sample (as normality) is

$$\frac{a_1-a_2}{a_1}\frac{A}{B}\times\text{normality of acid.}$$

Digestion time

With the procedure and quantities detailed above it was found that within 2 hr. about 95% of the sample had been digested. Thereafter the process was much slower and more than 4 hr. were required to complete it. Hence 5 hr. was chosen as a suitable time.

Diffusion time

The curves in Fig. 3 demonstrate the course of diffusion and absorption of ammonia at room temperature from a 0·33% solution of ammonium sulphate released by potassium metaborate as already described. The progressive addition of the other liquids required for the full procedure—water for washing the digestion tubes and the acid digestion mixture—greatly lengthen the diffusion time (Fig. 3). This is presumably due to the lowered alkalinity and perhaps also to the reduction in surface/volume ratio of the mixture. On this basis 7 hr. was chosen for the diffusion time at room temperature.

Performance for samples containing 1·0–10·0 μg. N

(*a*) *Titration only*. Table 2 summarizes three sets of titrations of 7 μl. N/10 HCl against approx. N/10 NaOH in the burette. The standard deviation was 0·2–0·4%. The standard deviation on the burette readings is of the same order when the titration is done in conjunction with the rest of the procedure (see Tables 3 and 4).

Table 2

7·0 μl. 0·1026 N-HCl titrated against approx. N/10 NaOH.

No. of readings	Mean burette reading	Standard deviation (%)
10	148·3	0·32
10	148·2	0·40
10	148·6	0·25

Table 3

7·0 μl. 0·329% $(NH_4)_2SO_4$. Standard acid 0·1026 N

No. of readings	Mean burette reading	Standard deviation (%)	Nitrogen found (μg.)	Nitrogen expected (μg.)	Standard deviation (μg.)
11	64·4	0·51	4·82	4·88	0·04
7	64·4	0·26	4·87	4·88	0·03
6	64·4	0·13	4·87	4·88	0·02

Table 4

Egg albumin solutions. Standard acid 0·1026 N

Vol. of sample (μl.)	No. of readings	Mean reading burette	Standard deviation (%)	Mean blank reading	Standard deviation (%)	Nitrogen found (μg.)	Nitrogen expected (μg.)	Standard deviation (μg.)
0·74	5	115·5	0·48	(2) 147·0	—	2·17	2·15*	0·04
0·70	7	119·4	0·20	(6) 143·8	0·17	1·79	1·77*	0·02

* Weight of nitrogen assumed to be dry weight of albumin/6·25 μg.

(*b*) *Diffusion-titration.* The results of three sets of nitrogen estimations on 7 μl. samples of 0·33 % ammonium sulphate are given in Table 3, the standard deviation being 0·02–0·04 μg. N.

(*c*) *Digestion—diffusion—titration.* Samples were taken from solutions of egg-albumin containing about 2·5 μg. N per μl., the exact weight of nitrogen being assumed to be dry weight of albumin/6·25. The results tabulated in Table 4 give a standard deviation of 0·02–0·04 μg. N.

(*d*) *Protein separation.* An attempt to assess the efficiency of the precipitation technique was made by estimating the nitrogen in albumin and ammonium sulphate solutions, separately and after mixing, with and without precipitation with trichloracetic acid. The following results were obtained:

	Mean nitrogen found (μg.) (4 samples of each)
Albumin solution only	1·95
Same plus added ammonium sulphate	6·86
Ammonium sulphate only	4·90
Albumin solution (by difference)	1·96
Albumin precipitated from the solution and washed	1·92
Same with ammonium sulphate added before precipitation	1·91

The apparent loss of nitrogen from the albumin sample after precipitation is not unexpected in view of the probable presence of amino nitrogen not precipitable by trichloracetic acid. But a more important point is the practical identity of the last two figures, which proves that the non-precipitable nitrogen is effectively removed by the washing procedure.

Performance for samples containing 0·1–1·0 μg. N

For our purpose it was not necessary to reduce the scale to this range. But, since we have used the same digestion technique as Brüel *et al.* (1946), who estimated quantities of this order, it was of interest to find whether the rest of our procedure could be adapted. We therefore did four diffusions with 7 μl. samples of 0·033 % ammonium sulphate using N/100 acid and alkali. A standard deviation of 0·006 μg. N was obtained. It is probable therefore that the complete procedure would give about the same degree of accuracy as the method of Brüel *et al.* (Table 1).

Table 5

Insect blood samples (0·74 μl.)

	Total nitrogen (mg. %)	Protein nitrogen (mg. %)	Non-protein nitrogen (mg. %) (by difference)
Sialis lutaria:			
Specimen A	1205, 1206	885, 853	336
Specimen B	665, 655	345, 307	334
Aeschna cyanea:			
Specimen A	286, 284	185, 174, 171, 191	104
Specimen B	365, 354, 368	245, 251, 233	119

Estimation of nitrogen in body fluids

To illustrate the performance with actual blood samples, Table 5 shows results obtained from the blood of two specimens of each of the insect larvae *Sialis lutaria* and *Aeschna cyanea*. The large individual variation in protein nitrogen is a characteristic feature.

SUMMARY

1. An ultra-micro modification of the Kjeldahl method is described whereby nitrogen can be estimated in a blood sample of less than 1 μl. volume.

2. This is simpler than previously described methods as regards apparatus and procedure.

3. The separate estimations of protein and total nitrogen are also introduced.

4. For samples containing 1·0–10·0 μg. N the standard deviation is 0·02–0·04 μg. N. Preliminary tests indicate that for 0·1–1·0 μg. N the standard deviation would be of the order of 0·006–0·008 μg. N.

The basis of this method was worked out in the Zoophysiological Laboratory in Copenhagen in September 1947. We are very much indebted to the Director, Prof. P. Brandt Rehberg, and to Dr E. Zeuthen for their hospitality and encouragement and to Dr H. Holter of the Carlsberg Laboratory, for giving us the opportunity for practical experience of their micro-Kjeldahl technique (Brüel *et al.* 1946). One of us (L. C. B.) was in receipt of a grant from the Travelling Fund of the Royal Society, and the research of which this forms a part is supported by a grant from the Leverhulme Trust.

REFERENCES

Bentley, G. T. & Kirk, P. L. (1937). *Mikrochemie*, **21**, 260.
Boell, E. J. (1945). *Trans. Conn. Acad. Arts Sci.* **36**, 429.
Borsook, H. & Dubnoff, J. W. (1939). *J. Biol. Chem.* **131**, 163.
Brüel, D., Holter, H. & Linderstrøm-Lang, K. (1946). *C.R. Lab. Carlsberg*, Ser. Chim., **25**, 289.
Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed. London.
Needham, J. & Boell, E. J. (1939). *Biochem. J.* **33**, 149.
Tompkins, E. R. & Kirk, P. L. (1942). *J. Biol. Chem.* **142**, 477.
Wigglesworth, V. B. (1938). *Biochem. J.* **31**, 1719.

THE SWIMMING OF WHALES AND A DISCUSSION OF GRAY'S PARADOX

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(With Plates 2 and 3 and Three Text-figures)

INTRODUCTION

The swimming of whales, whose well-authenticated maximum speed is 15 knots or more, is of interest not only to the biologist but also to those who would emulate this performance by artificial means. Fundamental to such a study is a knowledge of the swimming movement, which is not easily observed and has long been disputed. In this paper we give an account of this movement based on two separate lines of evidence: a morphological study (published in detail elsewhere) of the swimming muscles and associated structures of two species of whale; and an examination of a film of living porpoises for which we are greatly indebted to the Marine Studios, Florida, and extracts from which we publish here by their kind permission. Based on this account of the swimming movement it is possible to give a theoretical treatment of the dynamics of swimming, which may guide future experiment. Such a treatment involves a consideration of the drag of moving whales and this in turn leads to a discussion of Gray's Paradox: the big discrepancy which Gray (1936) showed to exist between the power apparently needed to overcome this drag at high speed, and the maximum power to be expected from the swimming muscles.

THE SWIMMING MOVEMENT

Photographic evidence

Pls. 2 and 3 show serial photographs of *Tursiops truncatus* from the film referred to above. It is a little unfortunate that the film was taken to show the birth of a young animal (seen projecting from the vagina) and so the photographs leave some doubt as to whether the movement is normal. The film itself, however, showed several other animals swimming about in the background, and although none of these provided a sequence suitable for reproduction, there was no doubt that they were swimming in the same way as the female on which the camera was concentrating.

The photographs show the tail and flukes moving as follows:

The *tail* moves up and down in a vertical plane about a centre of rotation just posterior to the dorsal fin, through an angle of approximately 70°.

The *flukes* move up and down in a vertical plane about a centre of rotation at the level of their insertion into the tail. Their movement is related to that of the tail as follows: when the tail is at the top of its stroke (and therefore momentarily at rest) the flukes are horizontal and so present the smallest possible resistance to motion in that plane. As the tail moves downward the flukes bend upward until at the middle of the stroke they are bent at their maximum angle to the horizontal. As the tail reaches the end of the downstroke the angle of the flukes is reduced, and they finally become horizontal again when the tail is momentarily at rest at the bottom of its stroke. During the second half cycle the movement is reversed: the tail moves upward, and the flukes move down, making an angle to the horizontal which increases to a maximum at the middle of the stroke and then decreases to zero. These details can all be followed by reference to Pls. 2 and 3.

The photographs show no sign of any lateral movement of the tail, and the absence of any such movement was fully confirmed by the original film, which contained shots of animals swimming directly away from the camera.

Anatomical evidence

In a study of the swimming muscles and axial skeleton of *Phocaena phocaena* and *Balaenoptera physalus* (Parry, 1949*a*), we show that these structures are developed to produce movements of the tail and flukes in the vertical plane, and that both the supra-spinal and infra-spinal muscle complexes are differentiated so that independent flexion of tail and flukes is possible. Thus the swimming movement seen in the Florida photographs is provided with an anatomical basis. And as it would be easier to fit the morphology to the film than the film to the morphology, it is relevant to state that the morphological study was completed, and the nature of the swimming movement was deduced, before the film became available to confirm our conclusions.

Discussion

In the next section we shall consider the hydrodynamic implications of the swimming movement just described; here we shall discuss the various accounts which have been given of the movement itself. Petersen's (1925) brief account, based on *a priori* considerations, agrees with the one given here. Other writers have limited themselves to the tail alone, without considering the movement of the flukes relating to the tail which we shall show to be of great importance. They mostly fall into two parties: those who believe that the tail moves simply up and down; and those who attribute to it a sculling motion. We suspect that this sculling theory arose from a remark of Scoresby's (1820) who, after saying of the Greenland Whale that 'the greatest velocity is produced by powerful strokes against the water, impressed alternately upward and downward'; adds that 'a slower motion, it is believed, is elegantly produced, by cutting the water laterally and obliquely downward, in a similar manner as a boat is forced along, with a single oar, by the operation of sculling' (p. 466). This is repeated by Beale (1839) and is to be found in a number of text-books and monographs, but the sculling theory derives little support from subsequent original observations in which either the vertical motion is asserted (Lillie, 1915; Slijper, 1936, referring to the film 'Abenteuer auf dem Meersboden'), or the lateral motion is not observed (Galloway, 1925; Petersen 1925; True, 1909; and photographs by Mcbride, 1940; and *The Illustrated London News*, 1910, p. 1001). Only Murie (1865) and Townsend (1914) give some support to sculling, the latter in very qualified terms after saying that the ordinary swimming motion is up and down. Reference should finally be made to the recent note by Woodcock (1948) where a vertical motion is implied and conditions in the clear waters of the Gulf of Panama were sufficiently good for the frequency of tail oscillation to be timed.

In a different category to the observations just referred to are the elaborate experiments of Shoulejkin and Stas, who believe that the swimming movement of dolphins consists of a circularly polarized wave passing down the body. Shoulejkin (1935) writes in Russian with an English summary in which the evidence for this theory is not given. In his text is a photograph from *The Illustrated London News* (1910, p. 382) of a school of dolphins taken from the forecastle of a ship, their bodies distorted into corkscrew shapes by the surface ripples; and another of a large metal container on land, through a hole in the side of which there protrudes the hind-end of a dolphin. If the evidence for the circularly polarized wave goes no further than what is implied by these photographs, it is not very convincing. Stas (1939*a*, *b*), on the other hand, claims to provide experimental evidence for Shoulejkin's theory by means of a recorder strapped to the back of a dolphin which was then allowed to swim to the end of a 100 m. line. The recorder consisted of a drum lying mid-dorsally just posterior to the dorsal fin with its long axis fore-and-aft bearing a pulley wheel over which passed a string. This, guided by another pulley wheel on either side, ran posteriorly along the lateral lines, and was fastened to the lateral points of a bracelet encircling the peduncle. Lateral movements of the tail thus rotated the drum, on which a record was scratched by a needle mounted on a carriage which was itself moved slowly backwards parallel to the axis of the drum by a small propeller. The record was calibrated in one dimension by displacing the tail known amounts to one side by hand; and in the other dimension by towing it through the sea at the same speed as the Dolphin had swum (70 cm./sec.). A single record is reproduced, showing an oscillation 6 cm. in amplitude and a frequency of 1·7 per sec., which Stas interprets as the horizontal component of Shoulejkin's circularly polarized wave; superimposed on which are much slower oscillations which he takes to be steering movements.

In a second experiment (1939*b*) the needle was not moved steadily across the drum on a carriage, but was controlled by a string which ran along the mid-dorsal line to the mid-dorsal point of the bracelet. The needle thus moved to and fro for vertical movements of the tail; and the drum turned from side to side for lateral movements of the tail. The record so obtained, which is not published, is said to have been an ellipse with half-axes in the ratio 1:1·5, the shorter corresponding to the lateral component of motion.

From the photograph in Stas's first paper showing the dolphin fitted with the recorder it appears that the swimming movement was not seriously impeded and the recorder was thus potentially capable of providing valuable information. It is therefore particularly unfortunate that the experiments were not described in more detail. In the first experiment we would like to know the position of the rubber band which, the author says, was used to tension the lateral string. For supposing, as we maintain, the swimming movement was in a vertical plane then, because the recorder was forward of the centre of rotation, each time the tail bent up or down the lateral string would become slack. And if the rubber band was at all asymmetrical in relation to this string then the slack would be taken up more on one side than the other, and so would rotate the drum and produce a record identical with that actually obtained in Stas's first experiment and interpreted by him as indicating *lateral* movement. This uncertainty would have been abolished if calibration records had been published showing the effect of displacing the tail (*a*) horizontally, and (*b*) vertically, whereas in fact we are not told that the latter calibration was done at all. The account of the second experiment is even more brief than the first, and there is no indication of how the dorsal string was tensioned. Neither are we told how clearly the record showed the predominant locus of the recording needle despite the numerous irregular marks which must have been made during turning and while the animal was being released and recaptured.

In view of these objections to Stas's experiments, we cannot accept Shoulejkin's theory in the face of the evidence of the Florida film, our studies of the anatomy, and the original observations of swimming whales discussed at the beginning of this subsection.

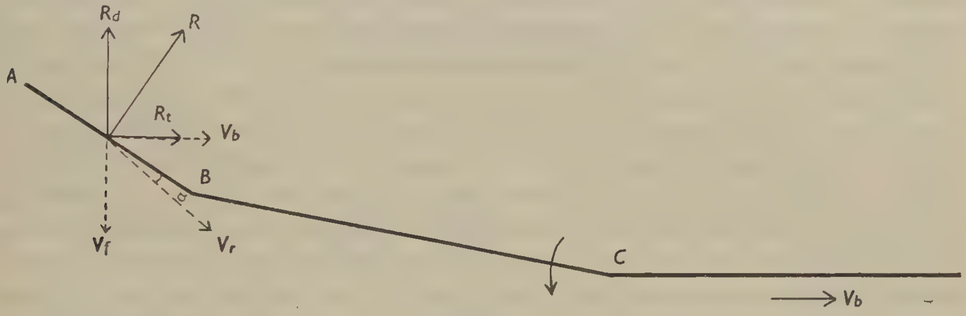


Fig. 1.

THE DYNAMICS OF SWIMMING

It is easy to show qualitatively that the swimming movement already described will produce a forward thrust. *AB* (Fig. 1) represents the fluke and *BC* the tail, which is being considered during the downward stroke. The whole system is moving forward with a velocity V_b. Thus at any moment the true velocity of the fluke through the water is represented by V_r, which is the resultant of the forward velocity V_b and the downward velocity of the fluke V_f. This resultant velocity will produce a resultant R of the water on the fluke, which can be resolved into a forward thrust R_t, which propels the system; and a drag R_d, which opposes the movement of the tail and against which the swimming muscles do work. It will also be seen that if the thrust is to remain positive throughout the downstroke, the water must be incident on the *underside* of the fluke—i.e. V_r must lie clockwise from *AB*. This means that the angle of the fluke must be adjusted in relation to its speed: as V_f decreases, *AB* must become more horizontal, until at the bottom of the stroke, when V_f is momentarily zero, AB will be exactly horizontal. Similarly, in the middle of the stroke, when V_f may be presumed to be maximum, AB will be inclined at its maximum angle.

A similar argument applies during the upstroke, when the water must be incident on the *upper* surface of the fluke.

Reference to the momentary condition illustrated in Fig. 1 will show that the flukes constitute an aerofoil moving through a fluid with a speed and direction represented by V_r, and with an angle of attack α. Now the forces acting on such a system are well understood when α is unchanging—this is the condition of an aeroplane wing in normal flight, and the forces are a known function of the angle, the shape of the wing, and its speed and area. A quantitative treatment of this method of propulsion is therefore possible if we can assume that the relationship between the angle of attack and the forces acting on the fluke is the same when the angle is changing as when it is constant. The little information that is available concerning aerofoils with oscillating angles of attack suggests that the above assumption is justified under certain conditions (see Farren, 1935; and Kuessner, quoted by v. Holst & Kuchemann, 1942). In the Appendix such a quantitative treatment is attempted, where we follow v. Holst & Kuchemann (1942) in integrating expressions for the instantaneous forward thrust and drag and so obtaining their mean values. Fig. 2 shows the mean thrust calculated in this way for a 180 cm. animal and plotted against speed for various arbitrary values for the frequency of the tail beat (n). But at any given speed the forward thrust must equal the 'parasite drag' of the body, and this provides an independent relationship between forward speed and thrust which therefore determines n for any speed and so provides an opportunity of checking the whole theory against empirical data. Now the parasite drag depends upon the amount of turbulence in the flow round the whale's body, which is at present unknown but is thought (see next section) to be small. Curves for fully turbulent and completely non-turbulent flow are drawn in Fig. 2, and their intersection with the family of curves relating speed, thrust, and oscillation frequency gives the following information:

For a 180 *cm. animal*

Frequency of tail beat ...	$\frac{1}{4}$	$\frac{1}{2}$	1	per sec.
Speed (assuming non-turbulence) ...	320	650	—	cm./sec.
Speed (assuming complete turbulence) ...	200	400	800	cm./sec.

It will be noted that over the range considered the speed is proportional to the frequency of tail beat.

The only observation at present available of the rate of tail beat during swimming is that recently published (since these calculations were made) by Woodcock (1948), who found that dolphins (size unstated) swimming beside a ship travelling at 10 knots had an oscillation frequency of 2/sec. In view of the necessarily approximate nature of our calculations, this observation is considered to provide evidence in favour of the theory of swimming here put forward.

GRAY'S PARADOX

The problem

Gray's Paradox is this (see Gray, 1936). During vigorous exercise a man is known to develop about 17 Watts of mechanical power per kg. of muscle. If this factor is assumed to apply to whales, then the power available in their muscles can be found as soon as the weight is known. But the power needed to drive a rigid streamlined body through the water, at a speed V, is given by the expression $\frac{1}{2}\rho AV^3C_f$ where C_f is the drag coefficient, ρ is the density of water, and A the surface area. Gray considered a porpoise and a dolphin swimming at about 15 knots, and found that the power met the demand only when the drag coefficient of laminar flow was used, whereas according to current theory the flow should be almost completely turbulent. When the coefficient of turbulent flow was used the demand exceeded the supply by a factor of seven.

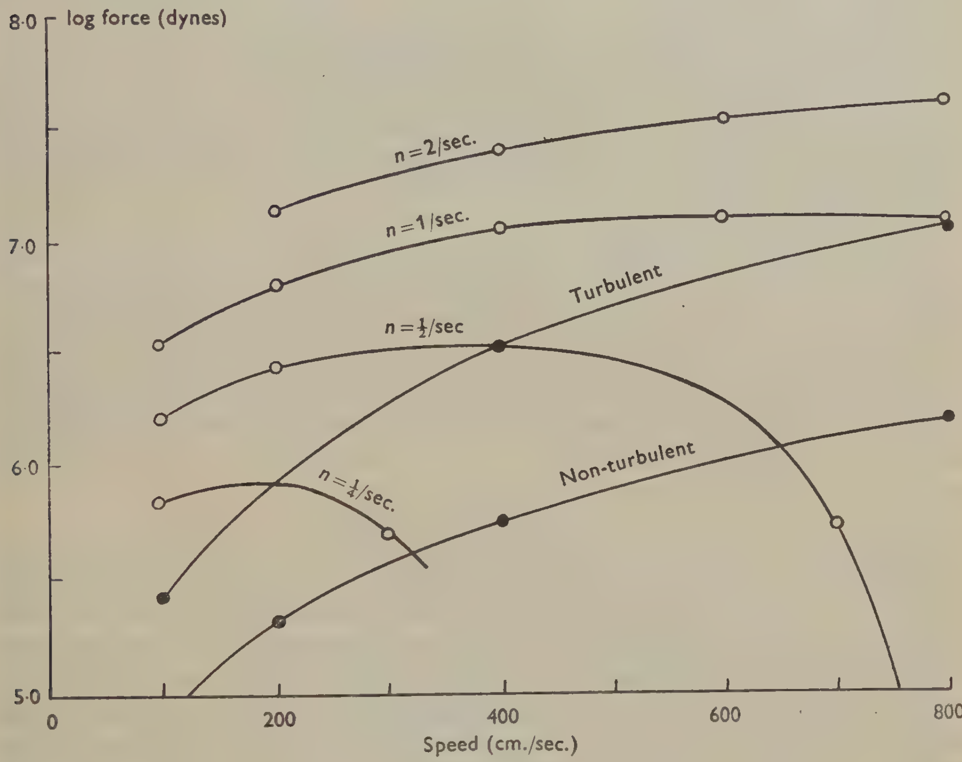


Fig. 2.

It will be seen from the above remarks that the supply is a function of the linear dimensions, whereas the demand is a function of the square. As the top speed of both large and small whales is about the same, it is the small ones that have the big power problem to solve. In fact, Gray's Paradox does not apply to whales over about 5 m. long.

Parasitic drag

Gray was inclined to resolve the paradox by suggesting that although the flow round a rigid body of the size and speed of a small whale was turbulent, that round and actively swimming whale might be almost completely laminar owing to the acceleration of the water due to the propulsive action of the flukes. Recent developments in fluid dynamics have modified earlier ideas concerning the transition between laminar and turbulent flow in a way favourable to Gray's hypothesis. It is now considered that the flow round a rigid body of the size of a small whale is unlikely to become turbulent until beyond the point of maximum cross-section (two-fifths of the length from the anterior end in the case of a whale-shaped body) and it is particularly in this posterior region, where turbulence would be expected in a rigid body, that the actively swimming whale differs from its rigid counterpart. As Gray suggested, the water here will be accelerating owing to the propulsive action of the flukes. Also the swimming movement will rhythmically lift and depress the tail out of the stream of water which has passed down the whale's body and is liable to become turbulent, and into undisturbed water which has not been in contact with the body at all.

Thus there is good reason to believe that the parasitic drag of an actively swimming whale is less than that of its rigid counterpart, and experimental evidence is now badly needed to establish whether or not this is so. But Gray's Paradox would only be resolved in the improbable event of all turbulence being suppressed; and even then the efficiency of the propulsive system, which for simplicity Gray assumed to be 100%, has still to be reckoned with. It is therefore necessary to examine the other side of the paradox and consider whether it is likely that whales can develop more than 17 W./kg. of mechanical power.

Muscle power

The figure of 17 W./kg. was derived from Henderson & Haggard's (1925) study of a rowing crew, and is approximately the greatest mechanical power which a man can develop without going into oxygen debt, when operating an efficient machine. Now it is well known that greater outputs are possible for short periods if an oxygen debt is allowed to accumulate. For example, we calculate from Dickinson's (1928) data on the bicycle ergometer (assuming a crank length of 18 cm. and a muscle mass of 10 kg.—see Vierordt, 1888) that a mechanical output of 40 W./kg. is possible under conditions of optimum efficiency. And so 17 W./kg. is not a limit set by the muscle itself, but by the supply of oxygen to the muscle (which determines the *total* power), and the efficiency of the system in which the muscle is operating (which determines how much of this total appears as *mechanical* power). If the rate of supply of oxygen to a whale's muscle, or the efficiency of the muscle during swimming, is greater than in Henderson and Haggard's rowing crew then a maintained mechanical power output of more than 17 W./kg. may be expected.

The efficiency of a whale's muscle during swimming is unlikely to exceed that of a man's muscle when the latter is operating such an efficient machine as a racing boat. But it is interesting to note that whereas the efficiency of a man's muscle during ordinary running is probably far from optimum owing to the high rate of contraction and the light load, that of a whale during fast swimming may be near or at the optimum owing to the much greater load and the slower speed of contraction.

Information about the rate of oxygen intake is given by Scholander (1940) and Irving, Scholander & Grinnell (1941), who find that the tidal air of *Tursiops* and *Phocaena* is about the same as the maximum found in man (on a weight to weight basis), and that the oxygen utilization is 2–2½ times as great. But this advantage is counteracted by the fact that whereas man can breath at a maximum rate of about forty breaths a minute a whale is unlikely to achieve half this rate even when swimming close to the surface.

We have briefly suggested elsewhere (Parry, 1948) that the rapidity with which whales recover their oxygen debt between dives might provide indirect evidence in favour of an unusually high rate of oxygen intake. This suggestion cannot be substantiated without data on the rate of underwater swimming. A porpoise has been observed blowing about three times between 4–5 min. dives. If it swam fast during these dives then under the most favourable hydrodynamic assumptions it would need to take up oxygen at a much greater rate than man in order to recover in three blows. But if it swam slowly then, owing to the dependence of power on the cube of speed, no special ability to absorb oxygen need be assumed even with fully turbulent flow.

Thus the possibility remains that whales can maintain a power output of over 17 W./kg. of muscle, but the best information we have about their respiration makes this unlikely.

SUMMARY

1. Whales swim by oscillating the tail and the flukes in a vertical plane. Tail and flukes move out of phase, the flukes leading on the tail by quarter of a wave-length. Photographic evidence is given and anatomical evidence referred to. Previous accounts of the movement are reviewed.

2. A theoretical treatment of the dynamics of swimming gives results in fair agreement with the only known observation of the frequency of tail-beat.

3. Support is given to Gray's suggestion that the drag of an actively swimming whale is less than that of a rigid model.

4. The possibility that whales develop more power (weight for weight) than man without going into oxygen debt is discussed. It is concluded to be unlikely.

I am most grateful to Prof. Gray for valuable discussion in connexion with the dynamics of swimming; to Mr J. H. Preston for information about recent work on the profile drag of aerofoils; to Dr R. H. J. Brown for drawing my attention to v. Holst & Kuchemann's theory of quasi-stable flow; and to Dr J. P. Harding for references to the Russian work on the swimming of whales. My indebtedness to the generosity of the Marine Studios, Florida, has already been noticed.

The work was done while I enjoyed a Senior Research Grant from the Department of Scientific and Industrial Research.

APPENDIX

Quantitative treatment of the dynamics of a swimming whale

V. Holst & Kuchemann (1942), in their studies of bird flight, have considered the dynamic characteristics of a wing of semi-span s, moving forward with a velocity v and at the same time oscillating sinusoidally at right angles to its course with a frequency n and amplitude a (expressed in terms of s). The angle of attack of the wing varies ϕ^c out of phase with the wing beat about a mean value α_o and with an amplitude α_1. Their method is to obtain expressions for the momentary forces C_x and C_z in the mean direction of movement and at right angles to it; and then to find the mean forces $\bar{C}_x$ and $\bar{C}_z$ by integration over one wing beat. To obtain C_x and C_z they use the expressions

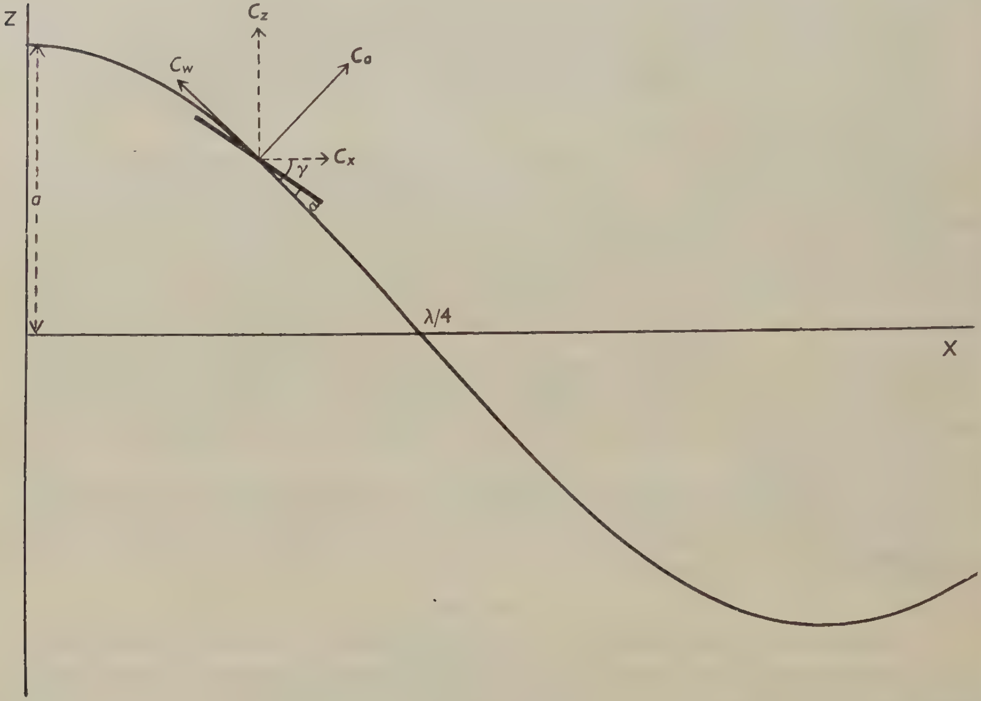


Fig. 3.

$$C_a = C'_a\alpha = \frac{2\pi\alpha}{1+(2/\Lambda)} \quad \text{and} \quad C_\omega = \frac{C_a^2}{\pi\Lambda} + \text{Profile Drag} \ - \text{where } \Lambda \text{ is the Aspect Ratio.}$$

These expressions are derived from the study of wings at fixed angles of attack. The basic assumption is therefore made that the flow round the wing of varying angle of attack, at any instant when the angle is α_t, is the same as it would be if the wing had remained indefinitely at α_t. The authors quote Kuessner as showing that this assumption is justified so long as the reduced frequency ($\nu = n \times s/V$) is not greater than 0·1 or 0·2.

The oscillating system considered by v. Holst & Kuchemann is similar to that of a whale's fluke and so their method can be applied if we make the basic assumption of stable flow.

Examination of Pls. 2 and 3 shows that the angle of attack is approximately 90° out of phase with the tail oscillation ($\phi=\pi/2$); and the mean angle of attack is 0 ($\alpha_0=0$). Thus the oscillation is symmetrical about the X-axis, and we have

$$\bar{C}_x=\frac{4}{\lambda}\int_0^{\lambda/4}(C_a\sin\gamma-C_\omega\cos\gamma)\,dx.$$

It can be shown that γ is fairly small, and so approximately $\sin\gamma=\gamma$ and $\cos\gamma=1$. Therefore

$$\bar{C}_x=C'_a\alpha_1\pi a\nu-\tfrac{1}{2}C'^2_a\alpha_1^2\frac{1}{\pi\Lambda}-C_{\omega p},$$

where $C_{\omega p}$ is the profile drag coefficient, and the mean force $\bar{C}_x$ is expressed in terms of $\frac{1}{2}A_fV^2\rho$, A_f being the area of the fluke, ρ the density of the fluid, and V the forward velocity.

Also
$$\bar{C}_z=\frac{4}{\lambda}\int_0^a C'_a\alpha dz=\frac{C'_a\alpha_1\pi}{4}.$$

Forward thrust. This is given by

$$\tfrac{1}{2}\rho A_fV^2\bar{C}_x.$$

From measurements of a porpoise we find that

$$A_f=3{\cdot}4\times10^{-2}L^2,\quad \Lambda=2,\quad a=1{\cdot}7,\quad s=0{\cdot}13L.$$

We have no means of knowing the maximum angle of attack (α_1) and so we will assume a likely value below the stall, i.e. 10° ($=0{\cdot}174^c$). The density of sea water at 10° round the British coasts is about 1·027; and the profile drag for a wing of aspect ratio 2 is 0·023 (Bairstow, 1939, 2nd ed. p. 449).

The forward thrust can now be expressed in terms of the body length (L), the oscillation frequency (n), and the forward speed (V).

$$\text{Thrust}=1{\cdot}75\times10^{-2}L^2V^2\left(\frac{0{\cdot}38Ln}{V}-0{\cdot}047\right).$$

Taking a whale of length $L=180$ cm. we can plot the thrust against the forward speed for different values of n (see Fig. 2).

Parasite drag. At any speed V, the drag of the body (parasite drag) must just equal the forward thrust. Now the parasite drag is given by

$$\tfrac{1}{2}\rho A_bV^2C_f,$$

where $A_b=\text{body area}=0{\cdot}39L^2$ (see Parry 1949*b*) and C_f is the drag coefficient. The drag coefficient depends on the Reynold's Number $\left(R=\frac{V\times L\times\rho}{\text{Viscosity}}\right)$ and the state of the boundary layer. In Fig. 2 we have plotted the drag for a laminar boundary layer ($C_f=1{\cdot}328R^{-\frac{1}{2}}$); and for a fully turbulent one ($C_f=0{\cdot}455\,(\log_{10}R)^{-2{\cdot}58}$, see Goldstein, 1938). Reasons are given in the text for thinking that the true drag lies nearer to the extreme of laminar flow.

Points at which the parasite drag curves cut the forward thrust curves indicate conditions of equilibrium, from which the results given on p. 28 are obtained.

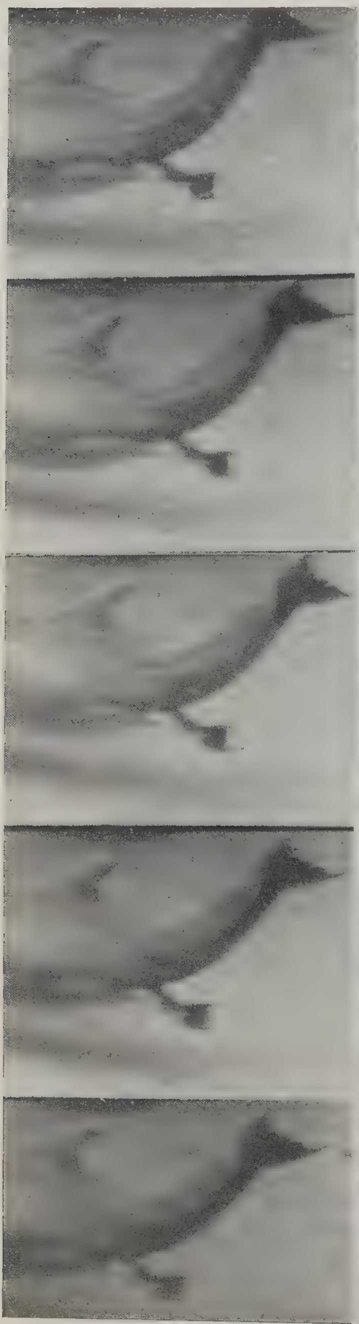
REFERENCES

BAIRSTOW, L. (1939). *Applied Aerodynamics*, 2nd ed. London.
BEALE, T. (1839). *The Natural History of the Sperm Whale*, 2nd ed. London.
DICKINSON, S. (1928). *Proc. Roy. Soc.* B. **103**, 225.
FARREN, W. S. (1935). *Aeronautical Research Council Reports and Memoranda*, no. 1648. London.
GALLOWAY, W. (1925). *Nature, Lond.*, **116**, 431.
GOLDSTEIN, S. (edit.) (1938). *Modern Developments in Fluid Dynamics*, vols. 1 and 2. Oxford.
GRAY, J. (1936). *J. Exp. Biol.* **13**, 192.
HENDERSON, Y. & HAGGARD, H. W. (1925). *Amer. J. Physiol.* **72**, 264.
v. HOLST, E. & KUCHEMANN, D. (1942). *J. R. Aero. Soc.* **46**, 39.
IRVING, L., SCHOLANDER, P. F. & GRINNELL, S. W. (1941). *J. Cell. Comp. Physiol.* **17**, 145.
LILLIE, D. G. (1915). British Antarctic (Terra Nova) Expedition, 1910. *Natural History Reports, Zoology*, **1**, 85. London.
MCBRIDE, A. F. (1940). *Nat. Hist. N.Y.* **45**, 16.
MURIE, J. (1865). *Proc. Zool. Soc. Lond.* p. 206.
PARRY, D. A. (1948). *Nature, Lond.*, **161**, 200.
PARRY, D. A. (1949*a*). *Proc. Zool. Soc. Lond.* **119** (in the Press).
PARRY, D. A. (1949*b*). *Quart. J. Micr. Sci.* **90** (in the Press).
PETERSEN, C. G. J. (1925). *Nature, Lond.*, **116**, 327.
SCHOLANDER, P. F. (1940). *Hvalråd. Skr.* No. 22.
SCORESBY, W. (Jun.) (1820). *An Account of the Arctic Regions.* **2** vols. Edinburgh.
SCHOULEJKIN, W. (1935). *Bull. Acad. Sci. U.R.S.S.* p. 651.
SLIJPER, E. J. (1936). *Capita Zool.* **7**, 1.
STAS, I. I. (1939*a*). *C.R. Acad. Sci. U.R.S.S.* **24**, 536.
STAS, I. I. (1939*b*). *C.R. Acad. Sci. U.R.S.S.* **25**, 668.
TOWNSEND, C. H. (1914). *Zoologica, N.Y.*, **1**, 289.
TRUE, F. W. (1909). *Smithson. Misc. Coll.* **52**, 325.
VIERORDT, H. (1888). *Anatomische, Physiologische und Physikalische Daten und Tabellen.* Jena.
WOODCOCK, A. H. (1948). *Nature, Lond.*, **161**, 602.
The Illustrated London News (1910). **137**, 382 and 1001.

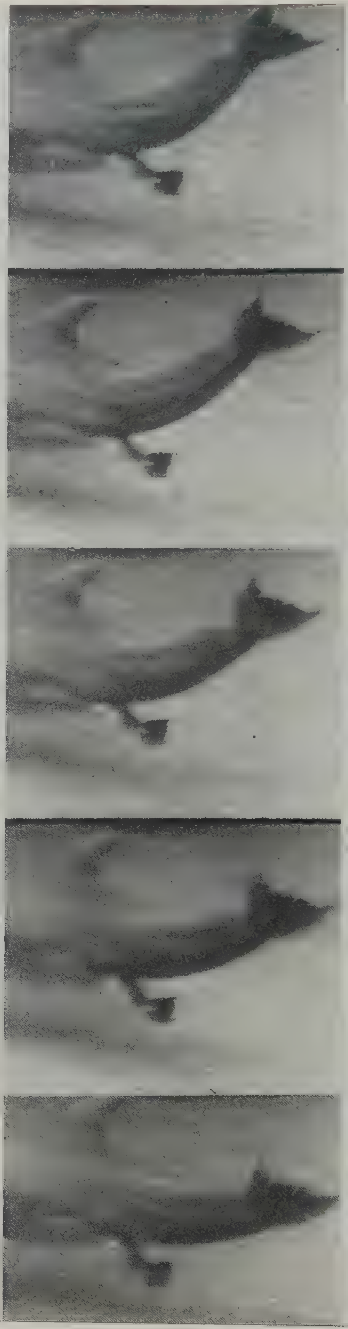
EXPLANATION OF PLATES 2 AND 3

Tursiops truncatus. Serial photographs showing one cycle of the swimming movement. Parturition is in progress and the young can be seen protruding from the parent's vagina. See text. (*From a film taken by Marine Studios, Florida, and reproduced by permission.*)

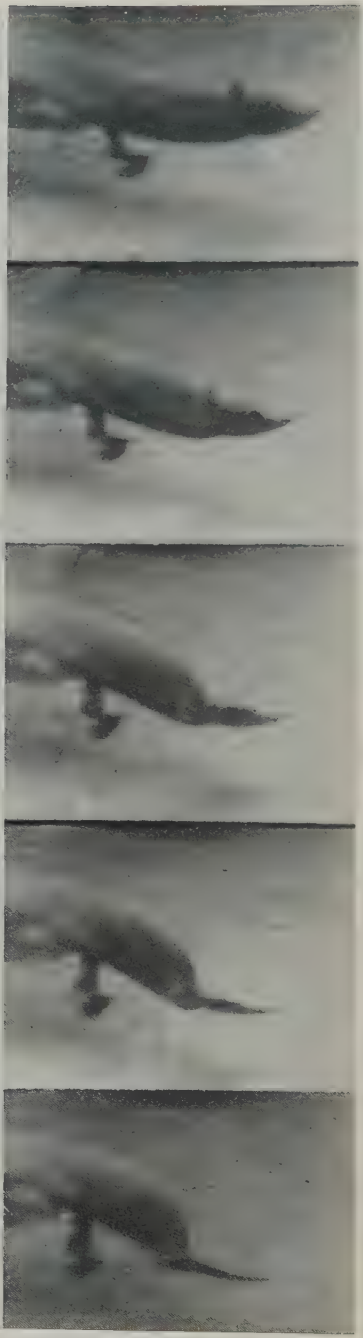
Figs. 1–5



Figs. 6–10

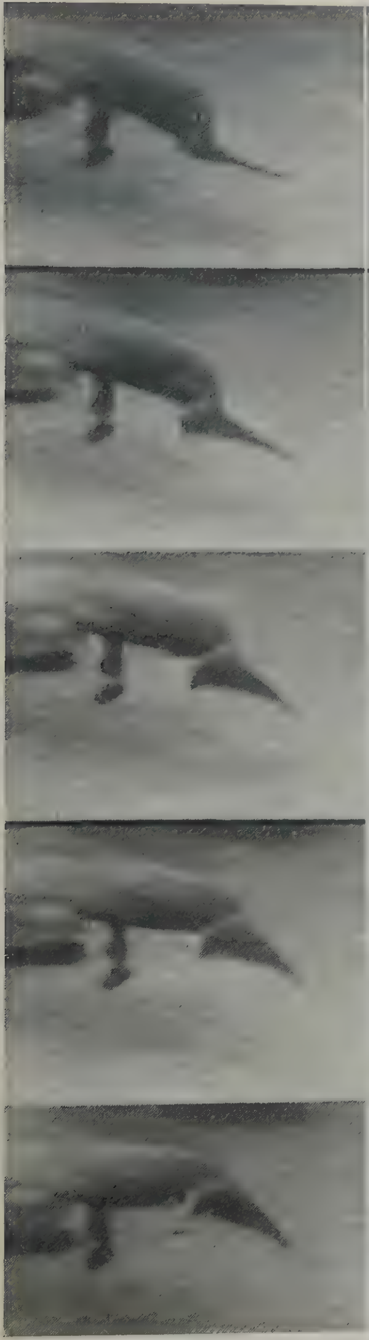


Figs. 11–15

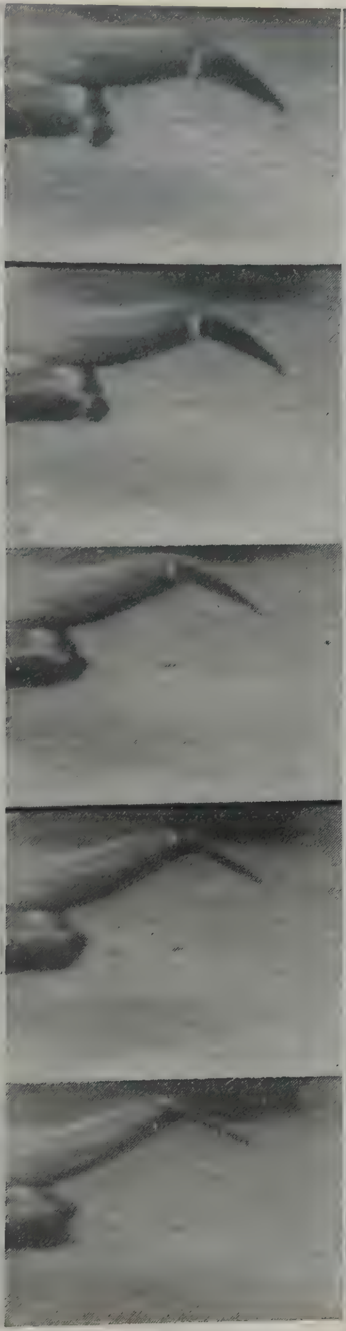


PARRY—THE SWIMMING OF WHALES, AND A DISCUSSION OF GRAY'S PARADOX

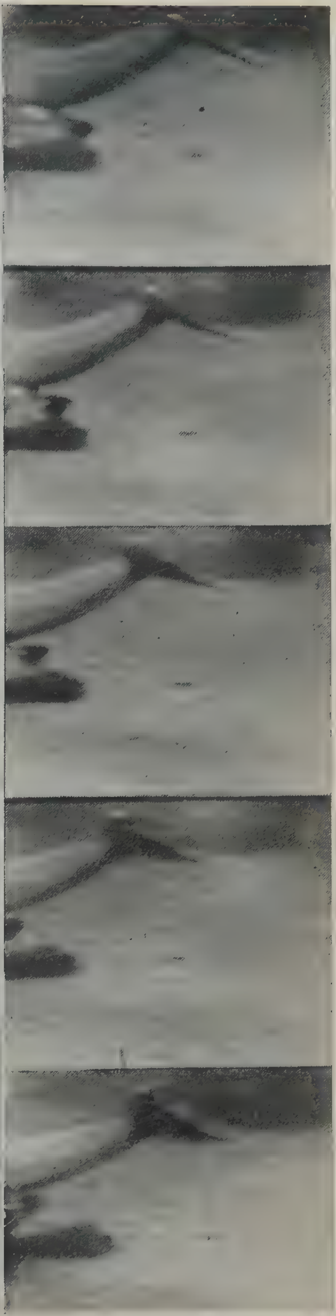
Figs. 16–20



Figs. 21–25



Figs. 26–30



PARRY—THE SWIMMING OF WHALES, AND A DISCUSSION OF GRAY'S PARADOX

SHAPE AND SHAPE TRANSFORMATIONS OF HEATED HUMAN RED CELLS

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(With One Text-figure)

The action of temperatures above 48° C. on the red cell is usually regarded as a classical example of the irreversible effects of injury, the lysis which follows being attributed to a surface membrane becoming permeable because its lipid components are melted or because its protein components become denatured. Recently it has been found that the effects are considerably more complex, and increasing emphasis is being placed on the role which fragmentation plays in the phenomenon of heat haemolysis. Ham, Shen, Fleming & Castle (1948) have pointed out that the fragmentation which precedes haemolysis results in the cells of the system becoming more and more spherical, so that the extent to which each cell can swell before its surface is stretched becomes less and less, and the tonicity in which it haemolyses becomes greater and greater; when there is sufficient fragmentation, indeed, human red cells will haemolyse even in an isotonic medium such as plasma. The tonicity-volume relations of cells which have lost one or more of these fragments during heating to temperatures in the neighbourhood of 48° C., however, are substantially the same as those of intact cells (Ponder, 1948*a*), and both cells and fragments show substantially the same resistance to haemolysis by saponin and by digitonin; these observations point to the osmotic properties and the properties which determine the resistance to lysins being shared by cell and fragment, and to the unit structure upon the integrity of which these properties depend being smaller than the intact red cell. The possibility that this is so suggests that something may be learned by examining the shape of the red cells and fragments more closely, since the ability to maintain shape and to undergo shape transformations are also properties which are usually thought of as associated with the red cell as a unit structure.

MATERIAL AND SPECIAL APPARATUS

The source of the red cells with which this paper is concerned is the heparinized blood of healthy donors, but the same methods can be used for studying the fragmentation of red cells from abnormal blood.

The cells are heated in one of three ways: in a small flat-bottomed flask immersed in a thermostatically controlled water bath, in a glass heating spiral immersed in the same water bath, or in an electrically heated chamber which fits into the moving stage of the microscope. The heating spiral is used to bring the cells to 48–52° C.

almost immediately and to keep them at the desired temperature for a known time. The chamber is used for the direct observation of individual cells before, during, and after heating.

The *spiral* is made of a piece of glass tubing about 2 mm. in internal diameter and about 200 mm. long, turned into a spiral of about 5 turns. It is mounted in a glass tube, open at the top, in order to protect it; the tube is immersed in a constant-temperature water bath, its lower end being brought outside the bath. It is filled through a small funnel attached to its upper end. The water bath can be set at any temperature in the neighbourhood of 50° C.

The double-walled *heating chamber** is rectangular in shape, measuring $11{\cdot}5 \times 6{\cdot}5 \times 2{\cdot}5$ cm. It is open above and below, and has inner walls of metal separated from its outer walls of Transite by 2 cm. A heating element, drawing about 1 amp. on the 110 V. circuit, is mounted between the outer and inner walls, and the current passing through it can be regulated by a variable resistance. The inner metal walls are perforated by small holes. The slide carrying the preparation forms the floor of the central space, which measures $7{\cdot}7 \times 2{\cdot}7 \times 2{\cdot}3$ cm., being kept just clear of the stage of the microscope by a narrow metal shelf projecting inwards from the lower edges of the inner metal walls. The central space is large enough to allow a high dry or an oil immersion objective to be focused on the cells of the preparation; considerable movement of the preparation is possible, and the slide is so close to the upper surface of the condenser as to allow critical illumination to be used if necessary (e.g., for photomicrography). Since the chamber is carried on the moving stage, the cells of successive fields can be inspected in the usual way. When the objective is in position and focused, the open top of the chamber can be covered with two pieces of mica, one on each side of the objective; more usually only one side is covered, the other being left open so that the cover-glass can be pressed on with a dissecting needle.

The temperature of the central space is indicated on a thermometer which projects into it. The chamber is heated by turning on the current for a minute or two. The rate of heating of the chamber is controlled with the resistance, and the observer soon becomes so familiar with the heating characteristics of the apparatus that he is able to control the temperature to within a degree or so. When it is desirable to know the temperature of the preparation more exactly, the latter is mounted on a slide which has a shallow well near one end; a single thermocouple projects into this well, which is filled with saline and covered with a cover-glass exactly as in the case of the preparation itself. Connected to a suitable galvanometer, this thermocouple measures the temperature in the well, which is presumably very nearly the same as that in the preparation.

The *extent of fragmentation* is measured by counting the objects seen on a haemacytometer chamber. The illumination used should be sufficiently high to prevent ghosts from being seen and included in the count. Care must be taken to allow the smaller fragments to settle on to the floor of the chamber and the precautions to be taken are, in general, the same as those taken in the counting of platelets. When there is extensive fragmentation, the cell suspension is diluted to a greater extent than when there is not. N is the ratio of the number of cells plus fragments in the heated system to the number of cells in the unheated system.

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* This heating chamber is made by Mr Paul Cutajar of the New York University machine shop.

SHAPE AND SHAPE TRANSFORMATIONS AFTER HEATING

(1) *Washed red cells in saline at 48° C.*

When heated to 48° C. in the heating spiral for between 2 and 8 min.*, and when examined soon afterwards between plastic surfaces under the microscope (high dry and oil immersion objectives) in the usual manner at room temperature,† washed human red cells in saline appear as a mixture of spheres, crenated spheroids, and flat cells with a distorted outline (Fig. 1 A); there are also a few fragments, but N does not usually exceed 1·5. The outline of the flat cells, which can be shown to be more or less typical disks by turning them up on edge, may present every kind of irregularity from simple scalloping to disruption into a fringe bordered with minute irregularly shaped fragments. Many of them show a clear central area which looks like a hole about 0·5 μ in diameter, and from which folds run radially towards a broken and fragmented region of the cell margin (Fig. 1 B, C, D); this radially arranged defect can be clearly seen in the cell when it is on edge. Judging by the way in which it behaves during disk-sphere transformations, the clear central area is probably a dimple in the material which forms the biconcavity.

The disk-sphere transformation between *slide and cover-glass* is typical except in so far as the cells are not perfect disks to begin with; some of them are spheres or spheroids, and so the shape transformation is not as striking as in preparations of unheated red cells. Perfect spheres are not as readily formed as in preparations containing unheated red cells; this shape transformation is notoriously difficult to control quantitatively, but the heating seems to result in an increased resistance to sphering. Reversal of the shape transformation occurs on the addition of plasma or of human serum albumin, the disks which reappear having clear central areas, radial defects, and scalloped edges, etc., just as they had before being sphered. The major irregularities on the cell surface, like the major irregularities on poikilocytes and on crenated red cells, are incorporated into the sphere as the disk becomes spherical, and probably reappear at the same sites on the surface when the sphere turns into the disk (cf. Furchgott, 1940).

The shape change produced by *lecithin*, as observed in preparations of heated red cells in saline between plastic surfaces, are very similar to the disk-sphere transformations between slide and cover-glass. As in the case of the latter, the shape changes produced by lecithin (distearyl lecithin sol in isotonic saline) do not occur as rapidly as in preparations containing unheated cells, but after a few minutes almost all the cells become perfect spheres. Addition of plasma, of serum albumin and of a number of other 'reversing substances' results in the reappearance of the same irregularly shaped disks, some with clear central areas, as are seen before the addition of the lecithin.

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* The fragmentation and other changes which occur in red cells after exposure to heat vary in the case of the cells of different healthy individuals, and vary very noticeably in the case of the abnormal red cells of certain types of anaemia. The specification 'heating to 48° C. for 2 min.', etc., is not to be taken too literally; the changes described usually occur when the cells are heated to this temperature for this time, but in the case of the red cells of another healthy individual, they may not occur until the heating has continued for a longer time or until the temperature has been raised to 48·5° C.

† No spontaneous shape change takes place when the cells cool. When plasma is run under the cover-glass to cool them quickly, an increased number of distorted disks appear; this is part of the shape change, from sphere to distorted disk, described in § (2).

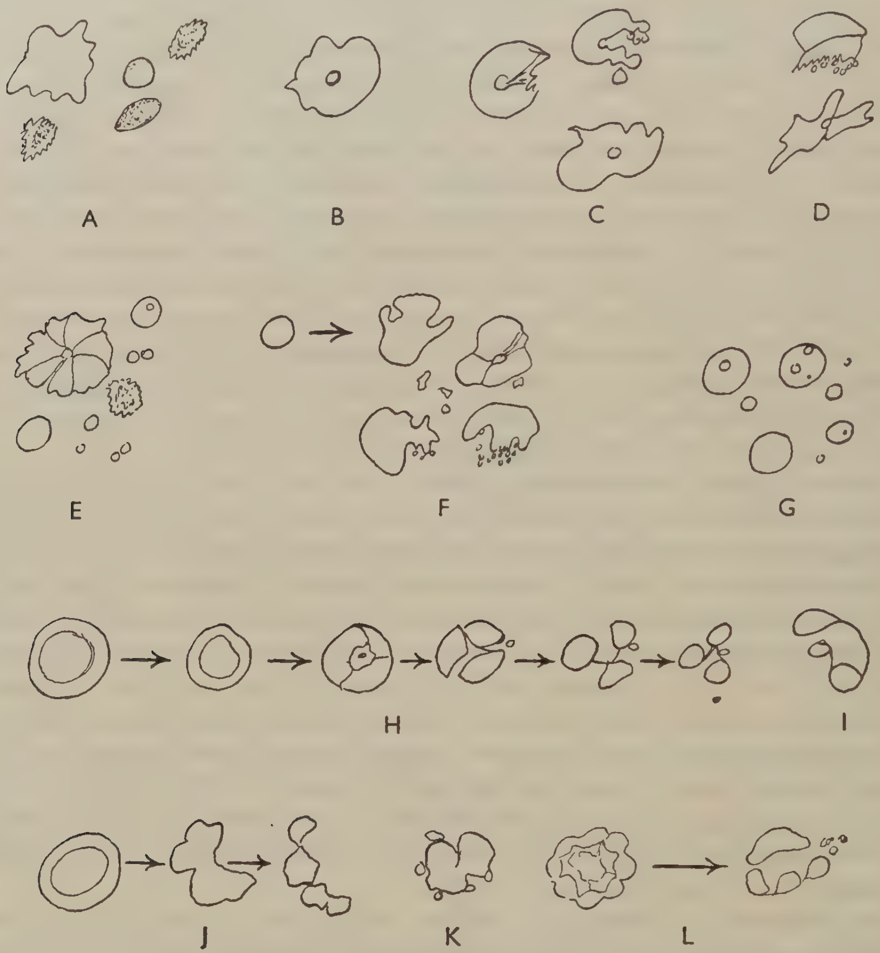

Fig. 1. Shape changes observed in heated human red cells. For explanation, see text.

The addition of *saponin* is followed by crenation; this soon disappears, and the cells become smooth spheres which later develop the peculiar appearance of the prolytic sphere and haemolyse. The behaviour of the clear central area and of the irregularities at the margins of the discoidal cells of the preparation can be better investigated by producing haemolysis with *rose bengal* (about 10^{-5} M). The cells of preparations between plastic surfaces become spherical soon after the dye is added, and the process can be hastened, so far as any individual red cell is concerned, by racking up the condenser of the microscope so as to increase the illumination incident upon the cell. As the disks lose their irregular outline and become more spherical, the larger of the irregularities may persist for some time before becoming rounded off and incorporated into the smooth sphere; the process resembles the transformation of a poikilocyte into a sphere (Ponder, 1942*a*). By selecting a cell with a clear central area, waiting until it is almost a sphere and then rolling it over so as to be able to inspect it from all directions, the clear central area can be seen to persist as a dimple on the cell surface until it ultimately becomes invisible in the perfect sphere. The clear central areas seen in the reformed disks after the addition of plasma to the preparation are presumably the same as the original clear central areas and as the dimples which disappear when the sphere is formed.

(2) *Washed red cells in saline at 50° C.*

When heated to 50° C. for 2 min. in the heating spiral, and when examined between plastic surfaces soon afterwards at room temperature, most of the cells appear as spheres surrounded by a large number of spherical fragments ($N = 2{\cdot}0$ to $3{\cdot}0$) of varying diameters (1·0 to 0·1 μ) (Fig. 1E). At least the larger of these fragments contain Hb; the preparation also contains ghosts of various sizes, the result of the 15 to 30% haemolysis which occurs in these heated systems. The few remaining disks are irregular in outline, some with clear central areas, some with radial defects, and some with scalloped margins. The occasional 'refractile body' (see below) turns out to be a fragment attached to the cell surface. The disks look peculiarly flat, and the 'rim', which normally looks distinctly thick and rounded, seems to be almost absent. On turning the disks on edge, however, it is clear that they are still biconcave, although they look thinner than unheated red cells do.

The shape transformation between *slide and cover-glass* and the transformation produced by *lecithin* are difficult to demonstrate because the preparations contain so few disks, and also because they occur very much more slowly than in preparations containing unheated red cells. The addition of 1% serum albumin, however, produces a remarkable effect which was first observed during an attempt to show that these disk-sphere transformations are reversible. Almost immediately after the serum albumin is added, many of the cells assume bizarre discoidal shapes; some of these disks have a clear central area, radial folds, and scalloping at the margins, but most of them are even more irregular in outline, 'pseudopods', veil-like fringes terminating in groups of minute granules, and other curiously shaped extensions appearing over as much as half the cell surface (Fig. 1F). Some spheres remain unchanged, and some turn into cup-shaped forms, but the preparation after the addition of the serum albumin would certainly be described as a preparation of bizarre and distorted disks rather than as one of spheres with an occasional disk, which it was before. Even the shape of the smaller particles is altered by the addition of the serum albumin; these small particles become oblong or irregular in shape instead of being substantially spherical as they were before.

The spherical cells observed before the addition of the serum albumin must accordingly be regarded as being the spherical end-product of a reversible disk-sphere transformation, the discoidal product being the bizarre disk. The effect of heating to 50° C. is probably first to produce irregularly shaped disks similar to those seen after heating to 48° C. but deformed to an even greater extent; these deformed (and also fragmented) disks then become spherical by an incorporation of their irregularities into the sphere (cf. the disk-sphere transformation of poikilocytes, Ponder, 1942*a*) so that they are seen as spheres even between plastic surfaces. The addition of serum albumin reverses this shape transformation, the sphere becoming the irregularly shaped disk once more, and it should be noticed that the shape of the fragments is affected in much the same way as the shape of the cells from which they were derived. Since the bizarre disks reappear on the addition of serum albumin (a well-known antisphering substance), it is likely that the spheres are the result of the heated cells having lost a material necessary for the maintenance of the (deformed) discoidal form. An anti-sphering substance, which converts the spherical forms of a preparation of unheated cells between slide and cover-glass back into disks, can be demonstrated to be present in considerable amounts in the supernatant fluid of a suspension of cells heated to 50° C. for 2 min. It is presumably derived from the heated cells.

Saponin and *rose bengal* produce sphering of the discoidal forms of the heated preparation, and all the cells and fragments haemolyse as spheres. There is no relation between the size of the cells or parts of cells and their resistance to lysis.*

(3) *Washed cells in saline at 52° C.*

When heated to 52° C. for 2 min. in the heating spiral and when examined between plastic surfaces shortly afterwards at room temperature, nearly all the cells appear as spheres surrounded by many Hb-bearing spherical fragments of varying sizes ($N = 3$ to 5). Some of the particles are so small that one cannot be sure whether they contain Hb or not, and the preparation also contains a considerable number of ghosts of various sizes corresponding to the 30–60% haemolysis which results from heating to this temperature. The preparation also contains a very few deformed disks, and the addition of serum albumin changes a few spheres into distorted disks, although the effect is much less noticeable than after heating to 50° C. The number of disks and of spheres transformable into disks, however, is so small that the slide and cover-glass and the lecithin shape changes can scarcely be demonstrated.

The spherical cells of these preparations appear to be unequally haemoglobinized. This is an appearance which can result from the cells being unequal in diameter and consequently presenting unequal paths for the absorption of light by Hb; it can also be the result of the cell lying above or below the focal plane of the optical system. There are so many cells and particles in the ordinary preparation that it is very difficult to pick out cells of equal diameter and to satisfy one's self that one is darker than another in all focal planes; the comparison is easily carried out, however, by making a simple modification of a 10 × ocular. A circle of black paper, with a central hole and another hole about 2 mm. from it, is cemented on the diaphragm which is built into the ocular in the focal plane of the eye-lens. These holes should be about 0·7 mm. in diameter; the central hole should be exactly centered. A field of the preparation is brought into focus with an ordinary ocular, an oil immersion objective, and a condenser at $\frac{1}{4}$ to $\frac{1}{3}$ cone; the modified ocular is then substituted, and a spherical cell is brought to the centre of the central hole. By rotating the ocular, other cells can be made to appear in the other hole, and each can be compared with the central cell; when the ocular has been rotated through 360° a new cell is brought into the central hole, and the process of comparison is continued.

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* I have observed, on several occasions, the fusion of two spherical fragments to form a larger sphere which haemolysed some minutes afterwards. This confirms a neglected observation of Auer (1932).

When this modified ocular is used, there is no difficulty in satisfying one's self that cells of substantially equal diameter are very unequally haemoglobinized. This means that the haemolysis observed in these systems is not all-or-none, and that there are more than two categories of cell—intact cells which have lost none of their Hb, and ghosts with a Hb content the same as that of the surrounding medium. Partial retention of Hb by ghosts has been demonstrated in the case of mammalian ghosts in hypotonic systems in which the ratio of cells to suspension medium is relatively small (<1 to 24, Ponder, 1942*b*; cf. Ponder, 1948*b*, in which the retention in Hb by ghosts is discussed in detail), and in the case of chicken ghosts in hypotonic systems (Hunter, Stringer & Weiss, 1940), but in these systems it was shown that the *average* Hb concentration in the ghost exceeds that in the surrounding medium; in the preparations under consideration, it can be shown that there are differences in the extent to which Hb is retained by the *individual* cells of the same system. The interest of this observation lies in its implications. The all-or-none loss of Hb from the mammalian red cell is observed in systems sufficiently hypotonic to produce lysis (Saslow, 1928; Parpart, 1931) when the ratio of cells to hypotonic medium is small, and one would expect Hb to be lost in this way from a balloon-like body containing Hb in solution. Both Saslow and Parpart have shown that the 'partial haemolysis' which other investigators observed in hypotonic media is traceable to experimental error, and even when retention of Hb has been demonstrated unequivocally (Ponder, 1942*b*; Hunter *et al.* 1940) it has been usual to suppose that the surplus Hb is adsorbed on the material of the cell surface or of an internal network. The unequal haemoglobinization of the spherical red cells of heated preparations cannot be accounted for in these ways, and is difficult to reconcile with the idea of the heated cell as a balloon-like body with Hb in solution. If it is suggested that the heating results in the Hb being 'fixed', it is necessary to specify in what sense it is 'fixed', for it can still leave the cell when haemolysis is brought about by osmotic means (Ponder, 1948*a*), by saponin, or by digitonin.

Most of the red cells heated to 52° C. for 2 min. contain one or more *refractile bodies*. These are variable in size (0·05 to 0·5 μ), not always round, and occupy a variety of positions in or on the cell (Fig. 1 G). The plane of focus in which they are best defined is rarely that of the cell diameter, which suggests that they are on the cell surface rather than in the interior. They seem to be in Brownian motion and to change position with respect to each other, but part, if not all, of this movement is probably due to the Brownian movement and rotation of the spherical cell itself. As a result of these changes in position, the bodies sometimes appear centrally placed, sometimes eccentrically placed, and sometimes placed at the very edge of the cell. They almost never appear as protuberances, and if they are associated with the cell surface rather than with the interior, they must extend inwards through the thickness of the surface ultrastructure rather than project from it.

The behaviour of these bodies during haemolysis is best observed by adding rose bengal to preparations containing washed red cells in saline, and accelerating the lysis by increasing the illumination. The refractile bodies disappear from view a fraction of a second before the cell begins to fade. When situated centrally, the refractile body often seems to roll off the cell and disappear; this apparent movement may be due to a sudden rotation of the cell itself. Nothing corresponding to the refractile body is seen in the ghost, except when it is situated at the very edge of the cell. A little roughness of the edge of the ghost may then be seen in a corresponding position.

The number, position, and behaviour of the refractile bodies suggest that they are situated at regions of the cell surface from which fragments have become detached and which are altered in structure as a consequence.

(4) *Observations in the heating chamber*

Cells in plasma (*glass surfaces*). As the temperature is raised, isolated disks begin to show either an irregular crenation or an increasing irregularity of their margins. Fragmentation begins so suddenly at about 50° C. that it is difficult to follow, but the process can be described as being of one of three kinds. (1) The discoidal cells become cup-shaped and smaller in diameter; a clear central area then appears in the region of the biconcavities. This area varies in size, is not always regular, and often has a minute haemoglobinized spot in its centre (as in the target cell). Quite suddenly, one or more radial defects run out from it through the material of the rim, and the cell breaks into one or more fragments which at first bear the same spatial relation to each other as they did when they were part of the same cell (Fig. 1 H). (2) 'Pseudopods', large or small, form at the margin of the discoidal cell, and defects run in from the bases of these through the material of the body of the cell, dividing it into two or more fragments of varying sizes (Fig. 1 J, K). (3) The rim of the cell becomes 'beaded' (as in the 'holly wreath' form of the sickle cell); the beads then tend to round up, and the cell breaks into a number of fragments (Fig. 1 L). The last of these fragmentation processes is uncommon; and the relative frequency with which the first and second occur seems to depend principally on whether the discoidal cells of the preparation are, or have a tendency to become, poikilocytes. Fragmentation by the inward extension of defects running from the bases of 'pseudopods' is apt to occur when the cells are derived from abnormal blood or when they have been stored for some days; these are conditions which favour the appearance of poikilocytes. Red cells from normal freshly collected heparinized blood usually fragment by the outward extension of a central defect.

The fragmentation occurs in the cells of rouleaux at the same time and temperature as that at which it occurs in isolated cells. The rouleaux break up into roughly spherical cells and fragments; these, however, tend to remain in alinement, much as they were in the rouleaux, until the cover-glass is pressed on: they are then scattered by being carried at random in the currents in the suspension medium.

Once formed, the fragments tend to round up, but they often retain their position with respect to each other for a long time, and can be seen to be connected by minute threads (Fig. 1 H, I). They haemolyse at about 58° C., some of the fragments derived from the same cell losing their Hb before others do.

Cells in saline between glass surfaces are, at first, spherical. As the temperature is increased, they become more glistening and smooth, but no fragmentation takes place at any time, even when the cells are made to move by pressing on the cover-glass. Ultimately the cells haemolyse, as spheres, at about 58° C. Cells in saline, in preparations between glass surfaces, can be converted into disks by the addition of 1 % serum albumin before they are heated. They then behave just like red cells in plasma on being heated, usually fragmenting by an outward extension of a central defect. The process of fragmentation is easier to observe in these preparations than in preparations of red cells in plasma, because of the absence of rouleaux.

Cells in saline between plastic surfaces fragment in much the same way as cells in plasma or in saline plus serum albumin fragment between glass surfaces. Thin plastic surfaces, however, tend to warp with heat.

DISCUSSION

In spite of a good deal of individual variation, it is clear that a number of regularly occurring phenomena are observed when human red cells are heated.

As regards its shape, the unheated red cell possesses shape-maintaining properties which can be modified in a variety of ways. The unheated cell, for example, maintains its normal discoidal shape when in plasma, but can be transformed into a sphere of substantially the same volume; in one of the disk-sphere transformations (slide and cover-glass) this shape change is due to the loss of an antisphering substance from the cell, and the restoration of the substance restores the discoidal shape. In another disk-sphere transformation (lecithin) the discoidal shape is restored by the addition of any one of a number of 'reversing substances'. The shape can be modified without the ability to undergo reversible shape transformations being modified, as, for example, in the case of the typical poikilocyte. This is what occurs in the case of the red cell heated to between 48 and 52° C.; the discoidal cell becomes an extremely irregular poikilocyte, but the irregularly shaped cell is still capable of undergoing reversible shape transformations. The irregularity can be accounted for by supposing that the molecular pattern upon which the discoidal shape depends has undergone an irreversible modification as a result of the heating; the modification, however, cannot amount to a complete destruction of the pattern, for the cell is able to pass from one metastable form, the poikilocyte, to another metastable form, the sphere, and back again. This capacity for undergoing transition from one metastable form to another becomes less as the cell is heated for longer times or to higher temperatures, but fragmentation of the cell does not destroy it; on the contrary, spherical fragments change into irregularly shaped fragments on the addition of anti-sphering substances just as the spherical cells of the same preparation change into poikilocytes. This means that the properties upon which the shape transformations depend are not necessarily dependent on the integrity of the red cell as a unit.

The effect of heat on red cell shape is complicated by the loss of an anti-sphering substance from the cells; this substance can be found in the suspension medium and can be replaced by adding 1 % serum albumin. Its loss, with the sphering which results, disguises the extent of the poikilocytosis which accompanies the fragmentation and which can be appreciated only after the addition of serum albumin and the reconversion of the spheres into irregularly discoidal forms. As already remarked, the addition of anti-sphering substance results in not only the cells, but also many of the smaller fragments, becoming irregularly shaped objects.

The loss of anti-sphering substance takes place at about the same temperature (49 to 51° C.) as that at which large amounts of potassium are lost by the cell and exchanged for some of the sodium of the suspension medium (Ponder, 1948*a*). At a slightly higher temperature, Hb is lost from the cells and fragments. It is not lost in an all-or-none fashion, spherical cells of many different degrees of haemoglobinization being readily distinguishable. Taken together with the loss of anti-sphering substance, the loss of potassium, and the shape changes preceding fragmentation, this step-wise loss of Hb suggests that the phenomena observed in the heated red cell are part of a process of disintegration of a plastic Hb-bearing 'solid' rather than manifestations of the breakdown of a balloon-like structure in which Hb is held in solution.

SUMMARY

This paper describes the shape changes and shape transformations observed in human red cells heated for 2 min. to 48, 50 and 52° C.

The first change observed is an irregularity of the cell; this is followed by sphering, but the spherical forms can be turned into irregularly shaped disks again by the addition of serum albumin. The sphering is accordingly part of a reversible disk-sphere transformation due to the loss of a (recoverable) anti-sphering substance from the cells. At a slightly higher temperature (50° C.) fragmentation of the cells occurs with the production of fragments which are also capable of undergoing something equivalent to a disk-sphere transformation. The properties upon which the shape transformations depend are therefore not necessarily dependent on the integrity of the red cell as a unit.

The fragments derived from the cell may be unequally haemoglobinized, and the Hb is usually lost in a step-wise, as opposed to an all-or-none, manner. Taken together with the loss of anti-sphering substance and an accompanying loss of potassium from the cells, the shape changes preceding fragmentation and the step-wise loss of Hb suggest that the phenomena observed in the heated red cell are part of a process of disintegration of a plastic Hb-bearing 'solid'.

REFERENCES

AUER, J. (1932). *J. Exp. Med.* **56**, 551.
FURCHGOTT, R. F. (1940). *Cold Spr. Harb. Symp. Quant. Biol.* **8**, 224.
HAM, T. H., SHEN, S. C., FLEMING, E. M. & CASTLE, W. B. (1948). *Blood*, **3**, 373.
HUNTER, F. R., STRINGER, L. D. & WEISS, H. D. (1940). *J. Cell. Comp. Physiol.* **16**, 123.
PARPART, A. K. (1931). *Biol. Bull. Woods Hole*, **61**, 500.
PONDER, E. (1942*a*). *J. Exp. Biol.* **19**, 220.
PONDER, E. (1942*b*). *J. Exp. Biol.* **18**, 257.
PONDER, E. (1948*a*). *J. Gen. Physiol.* **31**, 325.
PONDER, E. (1948*b*). *Haemolysis and Related Phenomena.* New York: Grune and Stratton.
SASLOW, G. (1928). *Quart. J. Exp. Physiol.* **19**, 329.

THE OSMOTIC RELATIONS OF THE EARTHWORM

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(With Two Text-figures)

I. INTRODUCTION

Interest in the water-relations of the earthworm appears to date from the work of Overton in 1904. In his paper 'Neununddreissig Thesen über die Wasserökonomie der Amphibien und die osmotischen Eigenschaften der Amphibienhaut' one 'thesis', the last, is devoted to the earthworm. He observed that an earthworm suspended in air lost water by evaporation and that when the partially dehydrated worm was placed in a U-tube of water with the mouth and anus above the surface, water was absorbed. He concluded that the surface of the animal was readily permeable to water in both directions and from this he argued that the water entering under osmotic forces must be got rid of by the excretory organs; such activity on the part of the nephridia he also claimed to have observed.

In 1925 Adolph & Adolph included the earthworm in an investigation of fresh-water animals which had as its object a study of the regulation of body volume. Their experiments consisted mainly in weighing worms at intervals after immersion in various solutions. No direct measurements were made of the osmotic pressure of the body fluids, but from the weight changes and from changes in the chloride content of the medium they calculated corresponding changes in internal osmotic pressure. They demonstrated that the initial weight changes, following transference from one medium to another, could be accounted for in general by the movement of water under osmotic forces, and that these initial changes were followed by slower changes under which the animal tended to return to its original weight at the beginning of the experiment. This work was continued by Adolph (1927), who gave the following picture of the earthworm's water-relations. Water enters by the skin and leaves by the intestine, there being no evidence that the nephridia are concerned: the exchanges of water which take place between the body fluids and external solutions are mediated entirely by the skin, the gut output being adjusted according to supply: water does not 'leak' into the worm under the influence of higher internal osmotic pressure because the skin is the seat of 'osmotic forces' which oppose this tendency.

Both Overton and Adolph observed that, on handling incidental to weighing, weight was lost by the worm. Wolf (1940) made a special study of this point and concluded that by far the greater part of this loss was due to the expulsion of fluid from the nephridiopores as originally suggested by Overton. Wolf did not commit himself to any comprehensive interpretation of water exchanges in the earthworm. Adolph (1943) accepted Wolf's conclusion that the loss of water was mainly via the nephridiopores without any further comment upon his own conclusions of 1927.

Prior to the appearance of Wolf's paper, Maluf (1939) described an investigation of the water-relations of the earthworm. Most of his experiments consisted in following changes in the weight of the worm, kept in water or in solutions, after various ligatures had been applied. He made the further important observation that the worm could take up chloride from very dilute solutions, even with both ends ligated, which implied that this active uptake was via the skin and not via the gut. Maluf took the view that water entered the worm by diffusion through the skin under osmotic forces, that it was stored in the gut and that it was got rid of through the nephridia and through the gut; he assumed that the fluids leaving these organs were hypotonic to the body fluids, but he did not verify this assumption by any measurements of osmotic pressure; he believed the gut to be of paramount importance in osmotic and volume regulation, as it were coming to the aid of the nephridia when the task was beyond their powers. In a later paper (1940), having measured the osmotic pressure of the gut fluids and found it to be only slightly less than that of the body fluids, Maluf retracted his earlier statement that the gut played an active part in osmotic regulation and returned to the view that the nephridia alone were responsible for getting rid of the excess of water and that they presumably must excrete an hypotonic fluid, as originally proposed by Overton.

The osmotic relations of the earthworm were also investigated by Stephenson (1945), who measured chloride content and conductivity of the body fluid and also changes in weight when the worms were kept in various solutions. He drew attention to the fact that volume changes on the one hand and chloride (and conductivity) changes on the other were not intimately correlated as Adolph had supposed. He further showed that, in addition to maintaining a higher internal chloride concentration in dilute solutions, the earthworm was able to maintain a lower internal chloride concentration in concentrated solutions, and this led him to the view that 'the earthworm is certainly no longer a fresh-water animal in so far as its osmotic relationships with the environment are concerned'—a view not shared by other workers in this field.

In a preliminary notice (Ramsay, 1946) it was reported that urine had been collected directly from the nephridiopores of the earthworm and had been found to be strongly hypotonic to the coelomic fluid. These preliminary observations have now been confirmed and extended and are described in more detail in the present paper. Although this work has had as its ultimate object an investigation of the physiology of nephridia, it was felt that this investigation could be undertaken with more confidence if some of the controversial issues regarding the earthworm's water balance could first be settled. Accordingly, some of the lines of study pursued by Stephenson were re-opened, with results now to be reported.

II. MATERIAL AND METHODS

For this work only worms of the species *Lumbricus terrestris* L. were used. I am indebted to Dr A. C. Evans of Rothamsted for instruction in the correct recognition of *L. terrestris* from among the other species with which it is liable to be confused.

For collection of the body fluids it was usual to have the worm lightly anaesthetized by short exposure to 15% alcohol and to pin it out on a board when it had become motionless and flaccid. The various fluids were allowed to rise into pipettes of pyrex glass, approximately 1 mm. in diameter, and coated with paraffin-wax on the outside. Collection of the coelomic fluid presented no difficulties. For collection of blood the pipette was inserted into the dorsal vessel and when this was successfully accomplished the blood would surge up into the pipette at each contraction of the dorsal vessel.

The collection of urine involved a more elaborate procedure. It is a simple matter to insert a pipette into the nephridiopore of an earthworm which is at rest, but an active worm loses little time in getting rid of it. The collection of urine from a single nephridiopore in sufficient quantity for analysis takes 1 hr. or more and there is reason to believe that changes occur in the urine of worms which suffer prolonged exposure to anaesthetics. It is not altogether easy, without extensive injury to the worm, to impose restraints which will keep it still. The method finally adopted was as follows. A ligature was applied around the clitellum and the posterior part of the worm was cut away. The anterior part was laid out on its side in a narrow shallow trough (Fig. 1). It was held at the posterior end by a pin passing through the clitellum and anteriorly a hook was passed through the peristomium and connected to a weight over a pulley. The effect of this was to keep the worm normally fully extended, but to allow it to shorten in response to a powerful contraction which, if resisted, would result in tearing out the attachments. With the worm thus lying on its side and extended, the dorsolateral openings of the nephridiopores were in a favourable position.

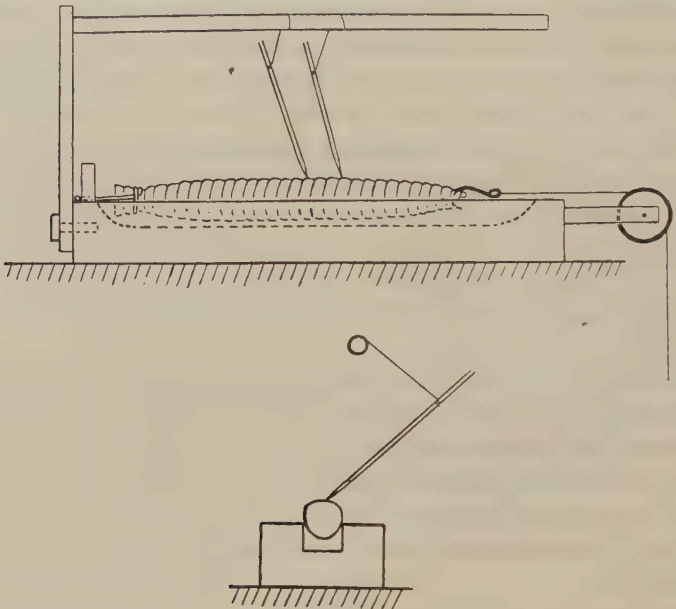

Fig. 1.

The pipettes were drawn to fine (15 μ) points from 0·5 mm. diameter pyrex tubing; they were varnished on the outside with bakelite so as to give a surface upon which a layer of wax could adhere firmly. The tips were dipped in molten paraffin-wax and a short length of very fine cotton was attached to the other end with sealing-wax. Above the trough was a brass rod, covered with plasticine. When a pipette was taken for use the cotton thread was laid over the plasticine and pressed into it. The pipette was then taken in forceps and (under a low power binocular) was inserted into a nephridiopore, where it remained suspended obliquely by the thread and pressing into the nephridiopore under its own weight. Since it conformed freely to the movements of the worm it was not readily dislodged. Several pipettes could be mounted in this way upon the same worm.

During the process of insertion the pipette was liable to become choked with mucus and when this happened it had to be discarded. After a successful insertion a small quantity of urine rose into the pipette, but the accumulation of urine was not continuous. Light scratching of the skin with a needle usually resulted in a local relaxation of both longitudinal and circular muscles and at the same time the urine could be observed to rise in the pipette. The preparation was covered with a perspex box (to prevent desiccation) and at intervals of about 20 min. the response to scratching was observed. The collection was continued for as long as urine continued to rise in the pipette in response to the stimulus, but after it had failed to do so on three successive occasions the pipette was withdrawn. A yield of 1 cu.mm. from a single nephridiopore was above the average; this was enough for two determinations of vapour pressure or for two determinations of chloride, or for one of each, but where smaller quantities were obtained they were usually pooled.

It was not generally feasible to carry out the analysis of fluids immediately after collection. For preservation over periods of a few hours or up to 2 days the two ends of the pipette were sealed off with paraffin-wax and the pipette was kept in a refrigerator. For preservation over longer periods the two ends were sealed off by fusion and the contents were sterilized by immersing the pipette in boiling glycerine (120° C.). This naturally had the effect of coagulating the proteins and the coagulum had to be separated by centrifuging. In the case of urine and coelomic fluid the coagulum is either non-existent or very slight. In the case of blood it is always very extensive and only a small proportion of clear fluid can be separated by centrifuging. The effect of this treatment upon the vapour pressure of coelomic fluid and blood and upon the chloride content of coelomic fluid was tested and was not found to be significant; in the case of blood no determination of chloride was possible on untreated samples.

Osmotic pressure was measured as vapour pressure by the Hill-Baldes thermo-electric method (Baldes & Johnson, 1939) and chloride by the micro-method of Wigglesworth (1937). In a few cases, where stated, use was made of a freezing-point method for osmotic pressure (see Table 2). The accuracy of these determinations varied according to the amount of fluid available for repetition of the measurements; where possible four measurements were made of vapour pressure and two of chloride on each sample and such results are believed to be accurate to ± 0·02 % NaCl.

Both osmotic pressure and chloride content are expressed as that concentration of NaCl%, which exerts the same osmotic pressure or has the same chloride content; and in this connection a 1% solution of NaCl is understood to be 1 g. NaCl dissolved in water and made up to 100 c.c. In converting osmotic pressures to freezing-point depression for comparison with the results of others the relation 1% NaCl≡0·585° C. △ has been used.

The media used in the experiments to be described were various concentrations and dilutions of frog ringer, of osmotic pressure varying between 0·0065 and 1·44% NaCl; tap water, equivalent to 0·02% NaCl, was also used. The vessels containing the worms were placed in a constant temperature room at 17° C. and aerated continuously. Under these conditions, and provided that the osmotic pressure of the medium lay between 0·01 and 1·0% NaCl, the worms survived well, less than 10% of deaths occurring in 3 weeks; as these limits were departed from the time of survival became progressively shorter.

III. RESULTS

(*a*) *Osmotic pressure and chloride content of the coelomic fluid in relation to the medium*

After a change of medium the osmotic pressure and chloride content of the coelomic fluid become practically constant at their new value in 3 or 4 days. Table 1 gives the results of an experiment in which worms were transferred from tap water to frog ringer≡0·75% NaCl and kept in this medium for 14 days before being returned to tap water. Four worms were taken at a time. Four determinations of vapour pressure and two of chloride were made on the coelomic fluid of each worm and the average values (accurate to ±0·02% NaCl) of these measurements are listed in the table. These values for individual worms are reproduced to show the range of variation from one worm to another. The average values for each set of four worms indicate the rate at which adjustment to the new medium proceeds.

Table 1. *Worms were placed in tap water in constant temperature room on* 6 *January* 1948: *transferred to frog ringer*≡0·75% NaCl *on* 20 *January* 1948: *returned to tap water on* 3 *February* 1948

Date	(1)		(2)		(3)		(4)		Average	
	O.P.	Cl	O.P.	Cl	O.P.	Cl	O.P.	Cl	O.P.	Cl
21. i. 48	0·75	0·45	0·74	0·43	0·77	—	0·78	0·44	0·76	0·44
23. i. 48	0·88	0·56	0·90	0·56	0·83	0·47	0·91	–	0·88	0·53
29. i. 48	0·90	0·49	0·94	0·54	0·97	0·53	0·97	0·46	0·94	0·50
26. i. 48	0·95	0·53	0·99	0·48	0·96	0·50	0·97	0·49	0·97	0·50
2. ii. 48	0·90	0·46	0·95	0·44	0·90	0·45	1·02	0·49	0·94	0·46
4. ii. 48	0·60	0·29	0·61	0·34	0·57	0·29	0·57	0·31	0·59	0·31
6. ii. 48	0·54	0·30	0·52	0·22	0·63	0·27	0·59	0·26	0·57	0·26
9. ii. 48	0·57	0·36	0·51	0·29	0·66	0·32	0·52	0·29	0·56	0·31
Controls in tap-water										
20. i. 48	0·50	0·27	0·54	0·24	0·56	0·26	0·55	0·26	0·54	0·26
2. ii. 48	0·66	0·31	0·59	0·27	0·56	0·29	0·59	0·28	0·60	0·29
9. ii. 48	0·52	0·25	0·52	0·23	0·54	0·25	0·58	0·25	0·54	0·24

Fig. 2 summarizes the results of various experiments with different media in which the worms were allowed to remain for at least 7 days before the coelomic fluid was collected for analysis. From this figure the osmotic pressure of worms kept in tap-water is seen to be 0·53 % NaCl. This corresponds to a freezing-point depression of $\Delta=0{\cdot}31^\circ$ C. which is exactly the value given by Adolph (1927) for worms kept in tap-water; it is not in good agreement with the results of Maluf who gives $\Delta=0{\cdot}45^\circ$ C. (1939) and $\Delta=0{\cdot}59^\circ$ C. (1940).

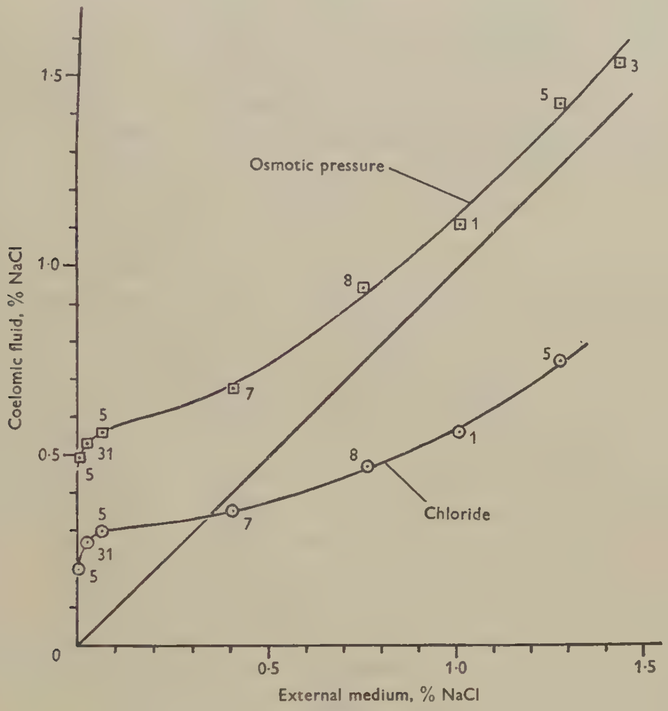

Fig. 2.

As the osmotic pressure of the medium is increased, the osmotic pressure of the coelomic fluid also increases and is always in excess of that of the medium. On the other hand, in media equivalent to 0·35 % NaCl or over, the chloride content of the coelomic fluid is less than that of the medium, as Stephenson found; the chloride values given in Fig. 2 are in very close agreement with the values given by Stephenson. It is clear that the earthworm has no powers of osmotic regulation in concentrated media, such as the chloride relations, unsupported by measurements of osmotic pressure, might seem to suggest.

(*b*) *Osmotic pressure and chloride content of the urine in relation to the medium*

The observations under this heading are assembled in Table 2. The values for osmotic pressure and for chloride show considerable variation from one collection to another, even from the same worm—more, in fact, than can be accounted for as

Table 2

			Urine		Coelomic fluid (from Fig. 2)	
Medium (% NaCl)	Date	Worm	O.P.	Cl	O.P.	Cl
Tap water ≡ 0·025	18. vi. 46	*A*	0·10	—		
			0·12	—		
	20. vi. 46	*A*	0·06	—		
			0·05	—		
	28. vi. 46	*A*	0·19	—		
			0·14	—		
	30. vi. 46	*A*	0·08	—		
	6. iv. 47	*A*	0·06	0·01		
		B	0·07	0·04		
		C	0·07	0·03		
	25. ii. 48	*A*	0·15	0·01		
		B	0·14	0·02		
			0·05	0·01		
		C	0·09	0·05		
	26. ii. 48	*A*	—	0·03		
		B	—	0·02		
	Average		0·10	0·02	0·53	0·27
Ringer ≡ 0·65	26. v. 47	*A*	0·24	—		
			0·28	—		
			—	0·12		
		B	0·14	0·04		
			—	0·05		
	27. v. 47	*A*	0·16	0·10		
			0·20	—		
			0·22	—		
			0·38	—		
		B	0·19	—		
			—	0·19		
	Average		0·23	0·10	0·85	0·43
Ringer ≡ 0·75	19. xii. 46	*A*	0·30	—		
		B	0·40	—		
	20. xii. 46	*A*	0·35	—		
	12. vi. 47	*A*	—	0·06		
	13. vi. 47	*A*	—	0·21		
	3. ii. 48*	*A*	0·41	0·22		
	4. ii. 48*	*A*	0·27	0·15		
	5. ii. 48*	*A*	0·17	0·13		
	7. ii. 48*	*A*	0·22	0·09		
		B	0·27	—		
	Average		0·30	0·14	0·95	0·48
Ringer ≡ 1·27	28. ii. 48*	*A*	1·38	0·50		
	3. iii. 48*	*A*	1·36	—		
	Average		1·37	0·50	1·40	(0·75)

* Freezing-point method used.

errors of analysis (which are ± 0·04% NaCl for osmotic pressure and ± 0·03% NaCl for chloride where only one measurement of each can be made). The average values, however, show quite clearly that in tap water the urine has an osmotic pressure of 0·10% NaCl, being strongly hypotonic to the coelomic fluid (as previously shown) and that the fraction attributable to chloride is so small as scarcely to be measurable. It is conceivable that the non-chloride matter in the urine represents the animal's normal nitrogenous excretion. From Wolf's results the urine production may be taken as 60 c.c./100 g. worm/24 hr. If nitrogen is present as two atoms of nitrogen per molecule of excretory matter, the total nitrogen excreted works out as 5·4 mg. N/100 g. worm/24 hr. Lesser (1908) gives a figure of 6 mg. N/100 g. worm/24 hr. But in experiments in which nitrogenous excretion is determined by analysis of the water in which the animal has been living one cannot be certain that all the nitrogen does, in fact, pass out by way of the nephridia. The view that the non-chloride matter in the urine may be accounted for as normal nitrogenous excretion is therefore put forward with reserve.

In media equivalent to 0·65 and 0·75% NaCl the urine, although more concentrated than in tap water, is still strongly hypotonic to the coelomic fluid, and of a total osmotic pressure of 0·3% NaCl about half is due to chloride which now makes its appearance in significant concentration. In media equivalent to 1·27% NaCl the urine seems to be almost isotonic with the coelomic fluid, but in view of the small number of observations this result must be accepted with caution.

It was very noticeable that the amount of urine obtainable from worms kept in frog ringer, especially in the higher concentrations, was much less than for worms kept in tap water. Collections from several nephridiopores had to be pooled and even then it was not always possible to determine both osmotic pressure and chloride on the same sample. For 3 or 4 days after the worms had been transferred to a concentrated medium, e.g. equivalent to 0·75% NaCl, no urine whatever could be collected, but after a fortnight enough was available to make the attempt worth while. In still higher concentrations, e.g. equivalent to 1·27% NaCl, worms seldom survived as long as a fortnight and in spite of much effort in this direction only two collections were obtained from worms in such concentrated media.

(*c*) *Osmotic pressure and chloride content of the blood in relation to the coelomic fluid*

The osmotic pressures of the blood and of the coelomic fluid were compared in thirty-one cases in which the worms had been freshly taken from soil, or had been kept in artificial media, or had been subjected to special treatment. In four cases the osmotic pressures were identical; in all other cases the osmotic pressure of the blood was slightly lower, the average difference being 0·053% NaCl. As already mentioned, the chloride of the blood can only be determined when the haemoglobin has been coagulated and removed; and it is difficult to separate clear fluid from the clot in quantities sufficient for analysis. In two cases only was the blood chloride determined; as compared with the coelomic fluid the blood chloride was found to be lower by 0·01% NaCl in one case and by 0·03% NaCl in the other.

The difference in osmotic pressure is significant in relation to the accuracy of analysis; the difference in chloride is barely so. It is conceivable that when water enters through the skin it first enters the blood-stream and then diffuses into the coelomic fluid, and if this were so the lower osmotic pressure of the blood might be accounted for. Attempts were made to abolish or exaggerate the difference by collecting these fluids during exposure to desiccation and shortly after the return of desiccated worms to water; but no significant changes in the osmotic difference were observed as a result of such treatment.

Particular attention was paid to this point since Bahl (1945) has shown that in *Pheretima* the osmotic pressure of the blood ($\triangle = 0{\cdot}40$–$0{\cdot}50^{\circ}$ C.) is considerably higher than the osmotic pressure of the coelomic fluid ($\triangle = 0{\cdot}285$–$0{\cdot}31^{\circ}$ C.). A difference of this order is astonishing and appears to be unique in the animal kingdom.

IV. DISCUSSION

A few animals normally living in fresh water are able to survive transference to sea water (e.g. the eel) and in such animals it is found that the body fluids are hypertonic to the medium when this is fresh water and hypotonic to the medium when this is sea water. But in most fresh-water animals, as the osmotic pressure of the medium is raised, the osmotic pressure of the body fluids rises also until the limit of toleration is reached; the body fluids are never hypotonic to the medium. In this the earthworm is no exception. Nevertheless, although the osmotic pressure of the coelomic fluid is always greater than that of the medium, the chloride of the coelomic fluid may be less than that of the medium, as is the case in media equivalent to 0·35 % NaCl or over. This is not without its parallel in other fresh-water animals; the larva of *Aedes aegyptii*, studied by Wigglesworth (1938), shows precisely this relationship. It is also characteristic of fresh-water animals that they eliminate urine which is hypotonic to the body fluids and are able to make good loss of salts by absorption from very dilute solutions; in the case of the earthworm the production of hypotonic urine, reported previously, has now been confirmed, and the ability to take up chloride from very dilute solutions has been demonstrated by Maluf (1939). By indirect methods Wolf (1940) has estimated the rate of urine production as approximately 60 % of the body weight in 24 hr., which may be compared with the corresponding figures of up to 30 % for fresh-water fishes and an average of 0·5 % for marine fishes (see Krogh, 1938). In all these respects the earthworm behaves like a fresh-water animal and there seems to be little reason for doubt on this matter.

It is perhaps worth while to point out that the ability of the worm to maintain the chloride of its coelomic fluid below that of the medium does not require us to postulate an active process of salt excretion against a concentration gradient. In media equivalent to 0·35 % NaCl or over salt will no doubt tend to diffuse in, possibly aided by the process of active uptake which is known to occur in more dilute media. But since the internal osmotic pressure is always greater than that of the medium, water will also tend to enter; and further, since living tissues are in general very much more permeable to water than to salts, it may well be that the net effect of this entry of salt and water is to dilute the coelomic fluid. Under such conditions the worm will be able to maintain the chloride of the coelomic fluid lower than that of the medium and at the same time will be able to excrete an hypotonic urine.

No important body of facts is in conflict with the view that under all the conditions herein considered (1) water enters passively through the skin with the osmotic gradient, (2) salt enters through the skin, either passively with the concentration gradient (as in concentrated media) or by active absorption against the concentration gradient (as in dilute media) and (3) an hypotonic urine is eliminated, involving an active process of secretion against a concentration gradient. It does not seem to be necessary to postulate any other active processes involving osmotic work. The outlines of the picture are attractively simple but it is unlikely that this simplicity will be maintained when the details come to be filled in. We assume, for example, that the difference in osmotic pressure is the force which causes water to pass through the skin; but Adolph has stated (1927) and re-affirmed (1943) that when a desiccated earthworm is allowed to recover in water 'the rate of intake increases many fold at deficits that do not even double the believed osmotic pressure of the body fluids'. Again, Stephenson has investigated the relation between body volume and the osmotic pressure of the medium. It appears that the body volume is a maximum in a medium equivalent to 0·35% NaCl and declines as the medium becomes more concentrated or more dilute. This observation is difficult to reconcile with a simple conception of the water relations such as has been outlined above.

These and other anomalies must be borne in mind, but until more facts are available it does not seem profitable to discuss them in detail.

V. SUMMARY

1. Osmotic pressure and chloride concentration have been determined for the coelomic fluid, blood and urine of earthworms kept in various saline media.

2. About 50% of the osmotic pressure of the coelomic fluid and of the blood can be accounted for as chloride. The blood is very slightly hypotonic to the coelomic fluid.

3. As the concentration of the medium is increased the osmotic pressure of the body fluids also increases and is always greater than that of the medium; the chloride increases proportionately, but is less than that of the medium when the latter exceeds 0·35% NaCl.

4. The urine is always strongly hypotonic to the body fluids except possibly in the most concentrated media (over 1·0% NaCl).

5. The osmotic relations of the earthworm are such as are characteristic of fresh-water animals generally.

I wish to record my thanks to Mr J. L. Parkinson for supplying me with two vapour pressure thermocouples and for advice on the construction of these instruments. I also wish to thank Dr S. Smith and Dr G. B. B. M. Sutherland, each of whom kindly lent a galvanometer. Nearly all the measurements of vapour pressure in this work were carried out by my assistant Miss J. Gukenbiehl.

REFERENCES

ADOLPH, L. F. (1927). *J. Exp. Zool.* **47**, 31.
ADOLPH, E. F. (1943). *Physiological Regulations.* Jacques Cattell Press.
ADOLPH, E. F. & ADOLPH, P. E. (1925). *J. Exp. Zool.* **43**, 105.
BAHL, K. N. (1945). *Quart. J. Micr. Soc.* **85**, 343.
BALDES, E. J. & JOHNSON, A. F. (1939). *Biodynamica*, no. 47.
KROGH, A. (1938). *Osmotic Regulation in Aquatic Animals.* Cambridge University Press.
LESSER, E. J. (1908). *Z. Biol.* **50**, 421.
MALUF, N. S. R. (1939). *Zool. Jb. Abt. Allg. Zool. Physiol. Tiere*, **59**, 535.
MALUF, N. S. R. (1940). *J. Cell. Comp. Physiol.* **16**, 175.
OVERTON, E. (1904). *Verh. phys-med. Ges. Würzburg*, **36**, 277.
RAMSAY, J. A. (1946). *Nature, Lond.*, **158**, 665.
STEPHENSON, W. (1945). *Nature, Lond.*, **155**, 635.
WIGGLESWORTH, V. B. (1937). *Biochem. J.* **31**, 1719.
WIGGLESWORTH, V. B. (1938). *J. Exp. Biol.* **15**, 235.
WOLF, A. V. (1940). *Physiol. Zoöl.* **13**, 294.

A NEW METHOD OF FREEZING-POINT DETERMINATION FOR SMALL QUANTITIES

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(With Two Text-figures)

I. INTRODUCTION

To a very large extent progress in the field of invertebrate physiology is a measure of progress in the technique of manipulating small structures and analysing small samples. Within this field the study of osmotic regulation is no exception. Krogh (1938) has summarized the known methods of measuring osmotic pressure, applicable to quantities of fluid of the order of 0·1–1·0 cu.mm. The freezing-point method now to be described is capable of dealing with quantities one thousand times smaller.

II. PRINCIPLE

The main difficulty of the orthodox freezing-point methods, when applied to small quantities, is the difficulty of avoiding undercooling. Drucker & Schreiner (1913) showed that this could be overcome by first freezing the sample and then warming slowly so as to observe (with a hand lens) the disappearance of the last crystal of ice; the temperature at which this happens is the freezing-point, provided that certain precautions have been taken. This method was used by Fritsche (1916), who determined the freezing-points of 1–3 cu.mm. samples of *Daphnia* blood to the nearest 0·01° C. As described by Fritsche the method is difficult, requiring care and practice—'die ganze Methode ziemlich individuell ist'—and does not seem to have been used by others. It is apparent, however, that the main difficulty of the method lay in the control of temperature, and that the apparatus was not such as to make temperature control at all easy. It was therefore decided to examine the possibility of developing the method along the lines of better temperature control while retaining the principle of observing the disappearance rather than the formation of ice. In addition, it was proposed to observe the ice-crystals with a microscope, so as to make it possible to work with smaller quantities, and to keep the sample under liquid paraffin, so as to avoid loss of water by evaporation.

III. APPARATUS

The apparatus is illustrated in Figs. 1 and 2. 30% alcohol at −5° C. from a refrigerator unit is circulated through the outer jacket which is separated by an air-space from the inner vessel, also containing 30% alcohol, in which the sample and the thermometer are mounted. This inner vessel is stirred by compressed air which is first passed through a cooling coil in the outer jacket; since moisture in the compressed air tends to freeze in the cooling coil and block it, it is necessary to bubble the air through 95% alcohol before it enters the apparatus. The alcohol in the inner vessel can be warmed by passing a current through a loop of nichrome wire. This current is adjusted to give the desired rate of heating by means of a variable resistor, and when rapid heating is required the variable resistor is shorted out by a key. When the current is switched off the temperature of the alcohol in the inner vessel falls slowly to −5° C. Rapid cooling is brought about by introducing cold alcohol from the outer circulation through a pipe reaching to the bottom of the inner vessel, excess alcohol being drawn off from the top by the suction of a filter pump.

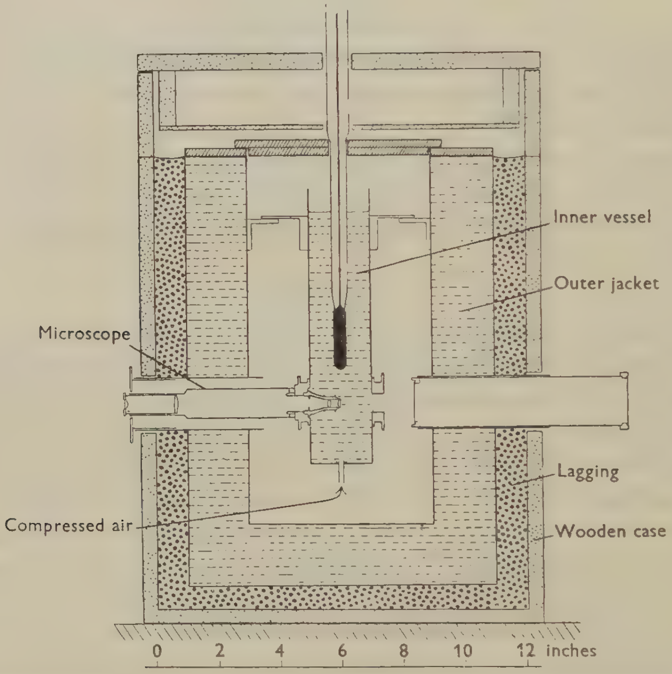

Fig. 1. To show the optical system and cooling system. The following features have been omitted for the sake of clarity: the coil on the compressed air supply, the pipes taking alcohol into and out of the inner vessel, the heating loop and the mounting carrying the sample.

A wide tube passing through the outer jacket allows a microscope to be mounted so as to observe the sample in the inner vessel. The objective, ⅔ in., is screwed into an adaptor in the wall of the inner vessel which is then lowered into position inside the outer jacket, and the tube of the microscope is then passed through and screwed into the other end of the adaptor. On the opposite side the inner vessel carries a window and light is admitted by another wide tube passing through the outer jacket.

The fact that the objective is rigidly fixed to the wall of the inner vessel makes it necessary to provide means of adjusting the sample to lie in the field of view and in focus. The arrangement for doing this is shown in Fig. 2 and is described in the legend to that figure. A fine adjustment to the focus is afforded by moving the microscope eye-piece in and out of the tube.

The thermometer used is a Beckmann, graduated in 0·01° C.

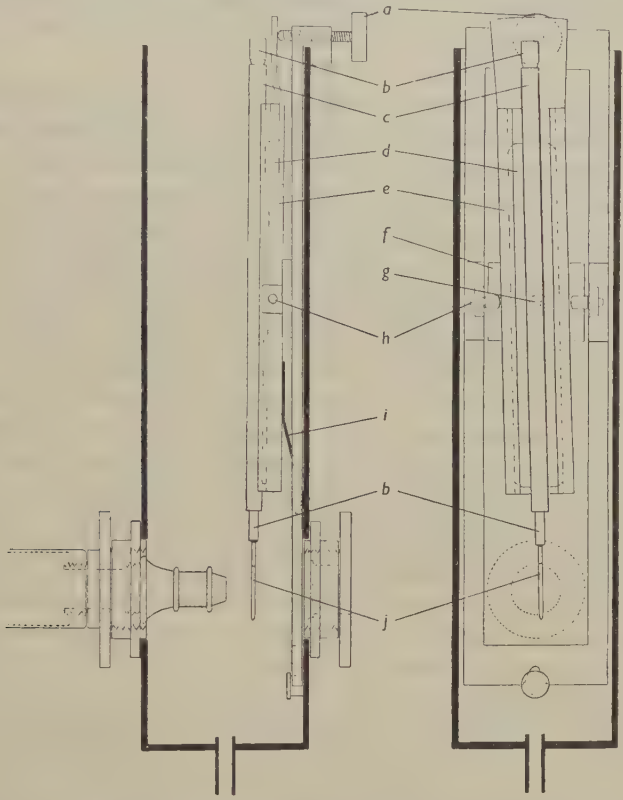

Fig. 2. To show the mounting by which the sample is adjusted in the field of view of the microscope. The sample is enclosed in the glass tube, *j*, which is fixed with sealing-wax to the brass rod, *b*. The brass rod, *b*, fits into the tube, *c*, which is fixed to the slide, *d*; this gives the up-and-down movement. The slide-guide, *e*, is mounted on the plate, *f*, and is free to move about the bearing, *g*; this gives the side-to-side movement. The plate, *f*, is carried on the bearings, *h*, and moved by the focusing screw, *a*, against the spring, *i*. The up-and-down and the side-to-side movements are friction-fitted.

IV. PROCEDURE

In order to present a concise and connected account of the procedure under the new method the reasons for certain of the steps have been omitted and are given at the end of this section under the 'Notes on Procedure', with numbers referring to the preceding text.

The pipettes are drawn from thin-walled 'Vitreosil' silica (see n. 1, p. 61) tubing of about 1 mm. external diameter. The collecting pipettes may be adapted in shape and dimensions to the problem in hand, but it is generally desirable that a collecting pipette should be drawn with a steep taper so that it is sufficiently rigid to be thrust into the tissues; the opening at the tip may be about 10 μ in diameter. The pipette is then fixed to a wider glass tube with sealing-wax and is connected through pressure-tubing to a mercury reservoir and screw-plunger. Mercury is driven to the tip and then withdrawn under liquid paraffin which is allowed to fill the tapering portion. The pipette is now ready for use. When a sample has been collected a further small quantity of liquid paraffin is drawn into the tip to prevent exposure of the sample to the air.

By reason of its steep taper the collecting pipette is not suitable for use in the determination of freezing-point (see n. 2). It is therefore necessary to transfer the sample to a freezing pipette which has a gentle taper from about 20 μ at the opening to about 70 μ at a distance of 1 cm. from the opening. The freezing pipette is mounted with sealing-wax upon a wider tube, connected to a mercury reservoir and screw-plunger in the same way as for the collecting pipette, and liquid paraffin is drawn into the tapering portion. Both pipettes are brought over the stage of a microscope, end to end and dipping into liquid paraffin. The tip of the collecting pipette is inserted into the tip of the freezing pipette and by manipulation of the screw-plungers the sample, or part of it, is transferred to the freezing pipette. A sample of volume $0{\cdot}1 \times 10^{-3}$ cu.mm. will fill the freezing pipette for a length of about 0·3 mm. It is then drawn up, followed by liquid paraffin, until in the wider part of the pipette its length is approximately double its diameter (see n. 3). The tip of the pipette is then removed from the liquid paraffin and is brought up against a drop of paraffin wax held in a loop of platinum wire. The wax is melted by passing current through the wire and a small amount of molten wax is drawn up into the tip of the pipette and serves to seal it (see n. 4). The pipette can now be removed from the wider tube on which it was mounted.

A piece of thin-walled glass tubing (see n. 5), about 1 mm. in diameter and about 4 cm. long, is sealed at one end and half-filled (see n. 6) with liquid paraffin. The freezing pipette is inserted into the open end of this tube and is pushed in as far as the taper will allow; it is then snapped off with forceps at this point and the end-portion is pushed right in until the part containing the sample passes beneath the surface of the liquid paraffin. The tube is then fixed with sealing-wax to the end of a brass rod (*b*, Fig. 2).

The brass rod is placed in the apparatus and adjusted so that the sample lies in the field of view. It is then removed and dipped for a few moments into 95% alcohol which has been cooled with carbon dioxide snow. Meanwhile, the temperature of the alcohol in the inner vessel is adjusted so as to be below the expected freezing-point of the sample. The sample, now frozen, is quickly transferred to the apparatus, the lid is replaced and the thermometer is inserted.

If the temperature of the alcohol is well below the freezing-point the sample appears more or less homogeneous. As the temperature is raised rapidly—at about 0·1° C./min.—by use of the shorting key, a sudden transformation is observed and the sample is seen to be made up of numerous round or oval ice-crystals with a small amount of liquid water. The temperature is allowed to rise rapidly until the volume of ice remaining is about one-fifth of the volume of the sample; the rate of heating must then be reduced. In the final stages a rate of heating of approximately 0·01° C.

in 2 min. has been found satisfactory (see n. 7). When the crystals become smaller than the diameter of the tube they float upwards and eventually the last crystal becomes hidden behind the curved surface of the meniscus (see n. 8). At this point the temperature is read to the nearest 0·005° C.

NOTES ON PROCEDURE

(1) Even with hard glasses such as pyrex it appears that substances from the glass dissolve into the sample and can appreciably lower the freezing-point.

(2) It is necessary to withdraw the sample far enough from the tip of the pipette to avoid any risk of its being boiled in the process of sealing with paraffin wax; in the steeply-tapered collecting pipette the sample forms a short column which breaks down and adheres to the wall after being withdrawn a short distance.

(3) If the sample formed a long narrow column the ice-crystals, floating to the top, would tend to cause dilution at the top as they melted, and in a long column diffusion might not be adequate to ensure uniform concentration throughout. The column is therefore made as short as is possible without risk of its breaking down.

(4) If sealing is omitted the sample is likely to be lost as a result of the sudden changes of pressure involved on removing the pipette from its mounting.

(5) Besides protecting the fine pipette containing the sample, this tube and the liquid paraffin contained in it fulfil important functions by virtue of their heat capacity. Notwithstanding the very rapid stirring which is maintained by the compressed air stream, mixing is never perfect. A small eddy, slightly warmer than the bulk of the alcohol, may strike the sample, and if this not protected by the wide tube its heat-capacity is so low that the last ice-crystal may be melted. The thermometer, measuring the bulk-temperature and unaffected by the eddy, records a temperature which is lower than that of the eddy which melted the ice and a false freezing-point is obtained.

(6) If the broken-off portion of the freezing pipette, containing the sample, were wholly immersed in the liquid paraffin it would be free to alter its position under gravity according to the inclination of the tube. This would be a nuisance if it happened after the sample had been adjusted to lie in the field of the microscope. With only 2 cm. of the tube filled with liquid paraffin one end of the pipette remains above the surface and is held firmly against the side of the tube by surface tension.

(7) The rate of heating must of course be so slow that the lag of the thermometer does not introduce any appreciable error. Owing to the latent heat of fusion of ice and the relatively poor thermal conductivity of liquid paraffin, equilibrium between ice and solution in the sample is established with surprising slowness, and as the size of the sample and its enclosing tubes is increased this effect is very much more significant than the lag of the thermometer.

(8) From simple geometrical considerations it can be shown that the volume of an ice-crystal as it disappears behind the meniscus is about 0·25% of the volume of a sample whose length is twice its diameter. The disappearance of the last ice-crystal behind the meniscus may therefore be accepted as the complete disappearance of ice from the sample.

V. ACCURACY

The accuracy of the method has been tested only so far as was necessary to establish its suitability for the investigation of the urine of earthworms (Ramsay, 1949). Results of repeated determinations on solutions of NaCl are given in Table 1. Each figure for freezing-point in this table is for a separate sample; the freezing-point of any given sample is normally reproducible to the nearest 0·005° C. From these results it appears that there is a reasonably linear relationship between △ and % NaCl, with a value of △/% NaCl = 0·60. If this relationship is accepted, the standard deviation of the observations is 0·00625° C. and the probable error is therefore 0·00427° C. or 0·007 % NaCl; but in view of the relatively small number of measurements, too small for proper statistical treatment, it would be better to put it that the probable error is less than 0·01 % NaCl.

The data given by Landolt-Börnstein (*Physikalische-Chemische Tabellen*, p. 811) show that the relation between concentration and freezing-point depression of solutions of NaCl is non-linear, but that over the range of 0–2 % concentration the errors involved in assuming a linear relationship are little more than 0·005° C. An exact comparison cannot be made since the Landolt-Börnstein data are in terms of g. NaCl/100 g. H_2O or g. NaCl/100 g. solution, whereas the data in Table 1 are in terms of g. NaCl/100 c.c. solution. Making allowance for this it appears that a value for the ratio △/% NaCl derived from the Landolt-Börnstein data would be somewhat lower, about 0·59. In view of these uncertainties it seems better to treat the new method as a purely empirical one, calibrating the freezing-point depression with solutions of known concentration and accepting whatever relationship gives the greatest consistency.

Table 1

Solution (g. NaCl/100 c.c.)	△ (°C.)	Volume (cu.mm. × 10^{-3})
0·50	0·295	Not recorded
	0·295	,,
	0·300	,,
	0·305	,,
1·00	0·595	0·20
	0·595	0·16
	0·605	0·06
	0·590	0·54
	0·605	0·04
2·00	1·200	0·54
	1·195	0·13
	1·205	0·14
	1·210	0·07
	1·205	0·29

Since the accuracies claimed for other methods are not defined in statistical terms a close comparison with the present method is not possible. Fritsche claims that his results are accurate to the nearest 0·01° C. but does not state whether this applies to repeated measurements on the same sample or to measurements on separate samples of the same solution. White (1928) states that his modification of the Barger method is capable of detecting the difference between 0·60 and 0·65 % NaCl, i.e. a difference of about 8 % of the total concentration. Keosian (1938), using the same method, claims that a difference of 2 % is significant. For the Hill-Baldes vapour-pressure method Johnson & Baldes (1938) claim that the method is 'sensitive' to changes of 2 mg. NaCl in 100 g. water; Fox & Baldes (1935) state that it is 'accurate' to within 1% of the total concentration. If we assume that the error of any single determination should not exceed three times the probable error, the 'accuracy' of the present method is 0·03% NaCl in concentrations of up to 2% NaCl and so is approximately 1·5% of the total concentration; its accuracy is therefore of the same order as the accuracy of the other methods.

The coelomic fluid and urine of earthworms contain very little protein, but as a matter of interest tests were carried out to see whether the method would be upset by protein in greater concentration. For this purpose human serum was used. No difficulty whatever was experienced in observing the freezing-point. Sixteen determinations of vapour-pressure by the Hill-Baldes method and five determinations of freezing-point were made with the following results:

Hill-Baldes method:	0·93% NaCl
Freezing-point method:	0·944% NaCl

The time required to carry out a determination of freezing-point by the new method is about 45 min., of which about 25 min. is taken up in transferring the sample from the collecting pipette to the freezing pipette and mounting it on the brass rod. In the course of routine work it is found convenient to have several brass rods available and to mount each sample as soon as it has been collected. At the end of the day the freezing-points of all the samples are determined, the average time per sample being about 15 min.

VI. DISCUSSION

As stated in the previous section, the accuracy of the method has been tested only so far as was necessary for a specific problem, and for this it proved to be entirely adequate. One feature of the results which calls for comment is the slight discrepancy between the freezing-point depression of a salt solution as measured by orthodox methods and as measured by the method now under consideration; but the reason for this discrepancy is not at present understood. This and other points will have to be cleared up before the limitations of the new method are defined. One such limitation will obviously be the solubility of constituents of the sample in liquid paraffin. However, it appears that the method is likely to have a useful range of application.

The apparatus is simple to construct. Its main disadvantage will appear to be the requirement of a refrigerator unit, an expensive item of equipment. It would have been difficult to develop this method without a refrigerator unit and it is certainly a great convenience to have it available; but all that is basically necessary is to have a supply of alcohol at about −5° C., and no doubt this could be provided by some system depending upon the conventional ice and salt mixtures.

The method works better with small quantities, e.g. 0·0001 cu.mm., than with larger quantities, e.g. 0·1 cu.mm. With large quantities the effect of the latent heat of fusion of ice in delaying equilibrium (see § IV, n. 7) is much more in evidence. A mass of small crystals of ice may appear to be in equilibrium when, in fact, the temperature is well above the freezing-point, the rate of melting being too slow to be appreciated. As the mass becomes smaller the rate of melting of the remaining crystals is increased and the last few disappear very quickly. One is therefore almost certain to overshoot the freezing-point if the temperature is raised continuously, unless the rate of rise is so slow as to require hours for the determination. In order to get an accurate measurement in reasonable time it is necessary to be able to cool rapidly when only one small crystal remains and then to adjust the temperature until the crystal neither increases nor decreases in size.

What is perhaps more interesting is to consider the possibility of working with still smaller quantities. The smallest quantity dealt with so far is $0{\cdot}04 \times 10^{-3}$ cu.mm. To go below this size a higher power of the microscope would be required and some refinement of the apparatus would be necessary for convenient working under, say, a $\frac{1}{6}$ in. objective. On purely optical considerations quantities of the order of 10^{-6} cu.mm. are a possibility, the size of the crystal disappearing behind the meniscus being of the order of $1\,\mu$ in diameter. A possible difficulty arises from the effect of surface tension at the ice-water interface, whereby crystals below a certain size become unstable. Dr A. E. Alexander, who kindly looked into this matter for me, finds that this effect will be negligible at crystal diameters greater than $0{\cdot}1\,\mu$, even in pure water or salt solutions; in the presence of protein or other surface-active substances the critical size will be less. More probably the difficulties to be encountered in dealing with smaller quantities will be those of manipulation and of contamination with impurities coming from the liquid paraffin and the silica.

VII. SUMMARY

1. A method is described for determining the freezing-point of small quantities ($0{\cdot}1 - 1{\cdot}0 \times 10^{-3}$ cu.mm.) of fluid.

2. Tested on solutions of NaCl of concentration up to 2% the probable error of the method is less than 0·01% NaCl.

3. The potentialities of the method are discussed.

I am indebted to Lord Rothschild for the loan of a refrigerator unit, without which the development of this method would not have been undertaken.

REFERENCES

Drucker, C. & Schreiner, E. (1913). *Biol. Zbl.* **33**, 99.
Fox, H. M. & Baldes, E. J. (1935). *J. Exp. Biol.* **12**, 174.
Fritsche, H. (1916). *Int. Rev. Hydrobiol.* **8**, 22.
Johnson, A. F. & Baldes, E. J. (1938). *Trans. Ill. Acad. Sci.* **31**, 198.
Keosian, J. (1938). *J. Cell. Comp. Physiol.* **12**, 23.
Krogh, A. (1938). *Osmotic Regulation in Aquatic Animals.* Cambridge University Press.
Ramsay, J. A. (1949). *J. Exp. Biol.* **26**, 65.
White, H. L. (1928). *Amer. J. Physiol.* **85**, 191.

THE SITE OF FORMATION OF HYPOTONIC URINE IN THE NEPHRIDIUM OF *LUMBRICUS*

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(*Received* 16 *October* 1948)

(With Three Text-figures)

I. INTRODUCTION

The structure of the nephridium of *Lumbricus* is known in some detail. The first adequate description is that of Gegenbaur (1853) and is correct in essentials. Goehlich's (1890) description is less accurate, as is pointed out by Benham (1891) who contributed further details of the structure of the nephridiostome and of the histology of the canal. Maziarski (1901, 1905) made a further study of histology; I have not had access to his second paper but it is summarized in the review by Meisenheimer (1910). Rosen (1911) re-described the nephridiostome but not altogether satisfactorily; the most recent and most accurate description is by Goodrich (1932).

Although these accounts are substantially in agreement there is some confusion in the names given to different parts of the nephridium and it is therefore necessary to give a brief description in order to define the terms to be used in this paper (see also Fig. 1).

The nephridium opens internally by the *nephridiostome* (Goodrich prefers this word to the more usual 'nephrostome'). Details of the structure of the nephridiostome are not relevant to the work described in this paper. The nephridiostome is followed by the *narrow tube* which is about 30μ in diameter, thin-walled and ciliated over certain regions. It traverses the first loop, then the second loop, then traverses the first loop a second time in the reverse direction. At the base of the first loop it is succeeded by the *middle tube*. The lumen suddenly enlarges to about 60μ or more; the walls become much thicker, bear cilia and have brown granules in the cytoplasm. The middle tube runs from the base of the second loop almost to its tip, gradually decreasing in diameter to about 20μ, and then opens into the *wide tube*. In the wide tube four regions can be distinguished. At its commencement it shows a wide dilatation, up to 100μ or more in diameter, and bends around the tip of the second loop; this is known as the *ampulla*. It is not histologically distinguishable from the succeeding region, known as the *proximal segment*, which is characterized by having thick walls with radial striations in the basal portion of the cytoplasm and what appears to be a brush border adjacent to the lumen. The wide tube traverses the second loop from tip to base and during this part of its course it decreases in diameter, its walls become thinner, and in histological features it shows a gradual reversion towards the narrow tube. This narrower portion of the wide tube may be spoken of as the *middle segment*, but it is not sharply demarcated from the proximal segment; it leaves the second loop and traverses the first loop. At the base of the first loop there is a sudden transition to the *distal segment*, about 70μ in diameter, with thicker walls showing the same basal striations as the proximal segment but lacking the brush border. The distal segment describes a wide sweep around the ventral part of the nephridium and enters upon the third loop. About half-way towards the tip of the third loop it opens into the *bladder*, but not at the point where the diameter of the canal appears to enlarge; the distal segment continues for a short distance in the wall of the bladder, opens out into a gutter and ends. The wall of the bladder is provided with a very thin epithelial lining surrounded by muscle fibres. The bladder completes the third loop and enters the body-wall under the ventral part of the nephridium to open eventually at the *nephridiopore* which may be either dorsolateral or ventrolateral in position. The whole organ is covered with a layer of peritoneal epithelium which forms a membrane uniting the three loops. The whole of the canal with the exception of the bladder is intracellular.

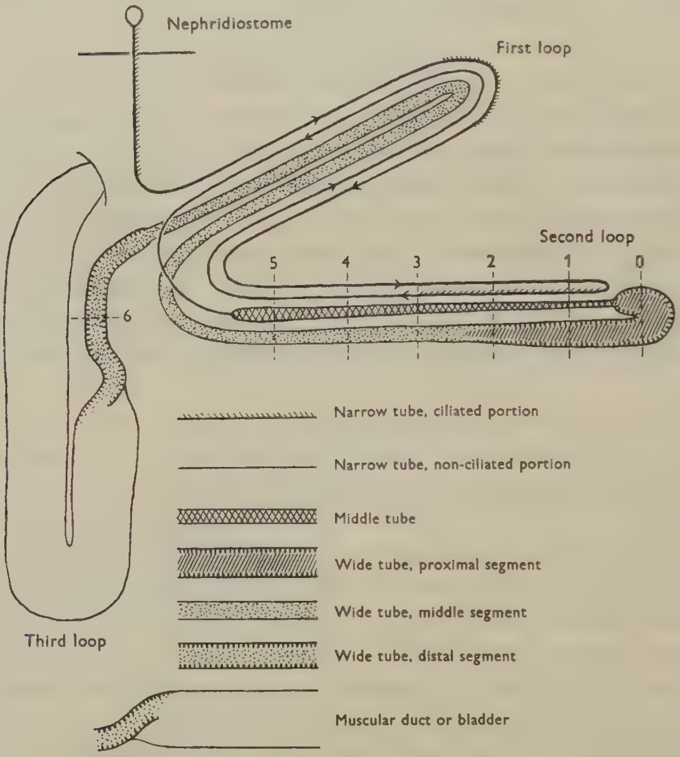
Nephridiostome
First loop
Second loop
5
4
3
2
1
0
6
Third loop
Narrow tube, ciliated portion
Narrow tube, non-ciliated portion
Middle tube
Wide tube, proximal segment
Wide tube, middle segment
Wide tube, distal segment
Muscular duct or bladder

Fig. 1. Diagram of the nephridium of *Lumbricus* (from the account by Meisenheimer). For reasons of clarity the loops are shown displaced from their normal positions. The numbers indicate certain positions at which samples were collected, and are referred to in the text.

About the function of the nephridium less is known. By collecting directly from the nephridiopores it has been shown that the nephridium eliminates considerable quantities of urine which is strongly hypotonic to the body fluids; the nephridium therefore plays an active part in water-balance (Ramsay, 1946, 1949*a*). In the latter paper the work of others bearing upon the problem of water-balance is referred to and discussed, and there is no need to summarize it here.

Apart from water-balance, physiological work is almost entirely restricted to the study of 'athrophagocytosis' which occurs in the middle tube. Contributions were made by Kowalevsky (1889), Schneider (1896), Cuenot (1898), Willem and Minné (1900) and Maziarski (1905); it was established that dyes and other substances, injected into the coelom, accumulated in the middle tube, but whether they reached that position directly from the coelomic fluid (elimination) or by being removed from the urine in its passage down the canal (resorption) could not be decided. Cordier (1933, 1934) carried out a more extensive series of observations, using colloidal particles of known size, and by analogy with similar work on the amphibian kidney he took the view that the process was one of resorption. Cordier's work will be considered in more detail in § IV of this paper.

The information available for other Oligochaeta is admirably reviewed by Bahl (1947). The only other genus for which comparable information is available is *Pheretima*, which Bahl himself has studied. *Pheretima* possesses three kinds of nephridia: (1) open septal nephridia, discharging into the intestine, (2) closed integumentary nephridia, discharging to the exterior, (3) closed tufted nephridia, discharging into the pharynx. These organs have been fully described and biochemical analyses have been carried out upon the blood, coelomic fluid and urine. From a comparison of these analyses Bahl concludes that the coelomic fluid enters the nephridial canal, that during its passage to the exterior potentially valuable constituents are resorbed and that an hypotonic urine is eliminated.

It is clear that Bahl's views about the nephridia of *Pheretima* have much in common with the Ludwig-Cushny filtration-resorption theory of the vertebrate kidney; and when one reflects upon the outstanding contribution made to this field by Richards and his associates one cannot but wonder whether similar methods of micro-sampling and micro-analysis might be applicable to the nephridium. In work of this kind the importance of choosing suitable material can hardly be overestimated. From the reviews of Bahl (1947) and of Goodrich (1945) it clearly emerges that *Lumbricus* offers many advantages as compared with other Oligochaeta such as *Pheretima*; in addition to being readily procurable, *Lumbricus* has nephridia all of the same kind, opening separately to the exterior, of relatively large size, accessible in dissection and adequately described. Accordingly, it was decided to proceed with the investigation of *Lumbricus* in an attempt to discover the site of formation of hypotonic urine.

With this purpose in mind a search was made for a method of measuring osmotic pressure on quantities of fluid considerably smaller than those required by existing methods. This led to the development of a method of freezing-point determination applicable to quantities of the order of 10^{-6} ml. or less. This method is described in another paper (Ramsay, 1946*b*). The present paper describes its application to the nephridium of *Lumbricus*.

II. MATERIALS AND METHODS

Throughout this work full-grown specimens of *Lumbricus terrestris* L. have been used. The worm is opened and pinned out in a dissecting dish under frog ringer in various concentrations and dilutions. The membrane connecting the loops of the nephridium is cut through as necessary and the whole organ excluding the nephridiostome is cut out and transferred to a microscope slide. Excess ringer is quickly drained and blotted and the preparation is at once covered with a layer of liquid paraffin which prevents it from drying up.

The collecting pipettes are drawn from thin-walled 'Vitreosil' silica tubing of about 1 mm. diameter with a steep taper and with openings varying from 5μ for the narrow tube to 20μ for the bladder. The pipette is fixed with sealing-wax to a wider glass tube connected through pressure tubing to a mercury reservoir and screw-plunger. The mercury is driven to the tip and then withdrawn under liquid paraffin which is allowed to fill the tapering portion. The glass tube carrying the pipette is mounted on a micro-manipulator.

The most serious difficulty is that of holding the organ steady while the pipette is being inserted. The nephridial canal is very tough and very extensible and penetration is only possible if it is held firmly and close to the point of insertion. In the case of collections made from the various parts of the canal represented in the second loop it is possible to press down upon the loop with a glass needle, held in a micromanipulator, and to insert the pipette as shown in Fig. 2*a*, *b*. In the case of the distal segment of the wide tube this method of holding is inadequate; the force which has to be applied to make the pipette penetrate is great enough to drag the tissues under the needle. In order to hold the tube steady a wool-fibre is tied around it, the knot serving to prevent the tissues from being drawn under the needle. In addition it is necessary to use a slide with a cover-glass cemented to it so that the edge of the cover-glass can be used to support the needle (Fig. 2*c*, *d*). In the case of the bladder it is also necessary to apply a ligature in order to retain the fluid when the organ is excised; the ligature is applied around the middle of the third loop which is then cut between the ligature and the base of the loop.

The process of penetration is observed under a ⅔ in. objective. When it has been successfully accomplished the tip of the pipette is very carefully adjusted so as to lie in the middle of the lumen. On releasing the pressure on the mercury the fluid rises very rapidly into the pipette and the walls of the canal collapse together. After the collapse of the walls the fluid ceases to rise in the pipette, showing that the seal is effective and that the outside medium does not enter the canal through the puncture. The pressure of the mercury is then raised slowly and carefully until the fluid in the pipette shows a tendency to return into the canal. The pipette is then withdrawn, raised into the paraffin layer, and paraffin is drawn in so that the sample is enclosed in a column of paraffin.

Satisfactory penetration cannot be made when there is insufficient pressure in the canal to keep the lumen open and this is more often than not the case with the middle tube and the distal segment of the wide tube. Even when penetration has been successful it is frequently found that the opening of the pipette has become blocked. Of all parts of the canal the ampulla is the easiest to deal with since the lumen is wide and the walls are transparent; about 80% of attempts on the ampulla are successful. The bladder by reason of its large size is easy to penetrate, but the muscle fibres in its wall often make it difficult to see the tip of the pipette and it is possible to imagine that the pipette has penetrated the bladder when in fact it has merely passed under a fold of the membrane which unites the loops. The narrow tube is more difficult, partly because of its smaller diameter and partly because its course, even in the second loop, is somewhat contorted; about 50% of attempts are successful. The middle tube and the distal segment of the wide tube are the most difficult, mainly because their walls show a readiness to collapse; only about 20% of attempts are successful, and in the case of the distal segment the time taken to apply the ligature means that fewer attempts are made. The difficulties of collecting from other parts of the canal are too formidable to be overcome by present technique.

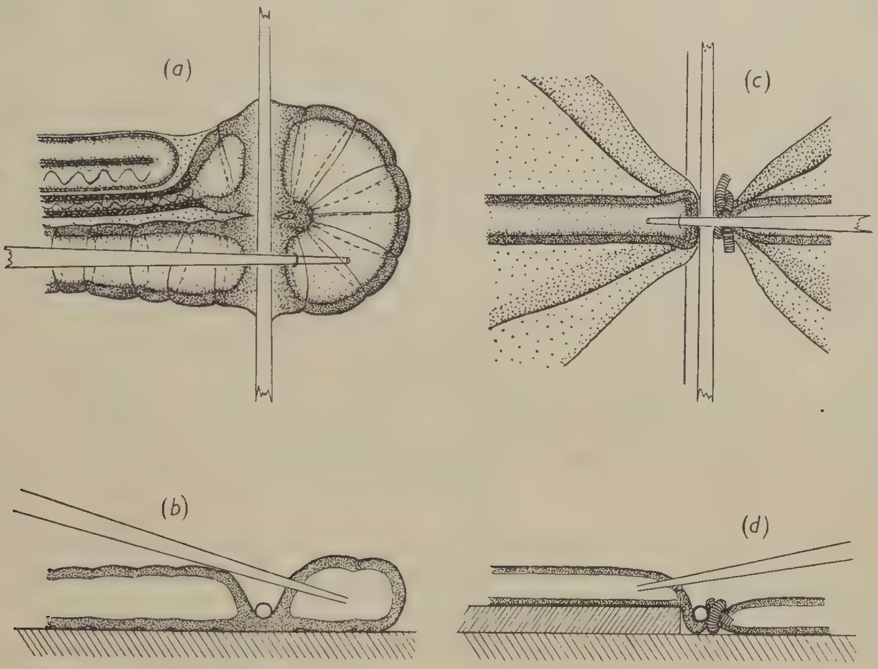

Fig. 2. Methods of collecting urine. *a*, *b*, from the ampulla; *c*, *d*, from the distal segment of the wide tube.

The volumes collected from various parts of the canal (excluding the bladder where much more could be obtained) varied from about $0{\cdot}1\text{–}5{\cdot}0 \times 10^{-6}$ ml. One determination of freezing-point was made on each sample, and the results are expressed in terms of the osmotically equivalent concentration of NaCl%. The probable error of each determination is 0·01% NaCl.

III. RESULTS

The results of the first series of collections are given in Table 1. As might be expected, the urine in the bladder is strongly hypotonic to the ringer; exceptions such as Serial 8 are probably due to faulty penetration as described under § II. The urine in the distal segment of the wide tube is also strongly hypotonic. The samples taken from the ampulla on 10 August 1948 are very clearly hypotonic and those

Table 1

Serial no.	Date	Worm	Dissection ringer (% NaCl)	Nephridium	Part of canal	Position of puncture	Sample (% NaCl)	Adherent ringer
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1	30. vi. 48	*A*	0·75	(*a*)	Bladder	—	0·05	Not recorded
2				(*b*)	,,	—	0·01	—
3				(*c*)	,,	—	0·11	—
4	5. vii. 48	*A*	0·75	(*a*)	Distal	6, base	0·17	—
5		*B*		(*a*)	,,	6 ,,	0·20	—
6		*C*		(*a*)	,,	6 ,,	0·16	—
7		*D*		(*a*)	,,	6 ,,	0·07	—
8	7. vii. 48	*A*	0·75	(*a*)	Bladder	—	0·71	—
9				(*b*)	,,	—	0·05	—
10				(*c*)	,,	—	0·02	—
11				(*d*)	,,	—	0·16	—
12				(*e*)	,,	—	0·16	—
13				(*f*)	,,	—	0·46	—
14				(*g*)	,,	—	0·27	—
15		*B*	0·75	(*a*)	Ampulla	0, tip	0·72	—
16				(*b*)	,,	0 ,,	0·52	—
17				(*c*)	,,	0 ,,	0·69	—
18				(*d*)	,,	0 ,,	0·56	—
19				(*e*)	,,	0 ,,	0·97	—
20				(*f*)	,,	0 ,,	0·61	—
21	8. vii. 48	*A*	0·75	(*a*)	Narrow, asc.	3, tip	0·87	—
22				(*b*)	,, ,,	3 ,,	0·79	—
23				(*c*)	,, ,,	3, base	0·79	—
24				(*d*)	,, ,,	3 ,,	0·77	—
25	9. vii. 48	*A*	0·75	(*a*)	Narrow, asc.	3, tip	0·71	—
26				(*b*)	,, ,,	4, tip	0·76	—
27				(*c*)	,, ,,	4 ,,	0·69	—
28				(*d*)	Narrow, des.	3, tip	0·76	—
29				(*e*)	Narrow, asc.	1, tip	0·80	—
30				(*f*)	,, ,,	5, tip	0·74	—
31	10. vii. 48	*A*	0·75	(*a*)	Ampulla	0, tip	0·36	—
32				(*b*)	,,	0 ,,	0·32	—
33				(*c*)	,,	0 ,,	0·34	—
34				(*d*)	,,	0 ,,	0·33	—
35				(*e*)	,,	0 ,,	0·36	—
36				(*f*)	,,	0 ,,	0·32	—

In this table, and in Table 2, the capital letters (column 3) are used to indicate different worms, the small letters (column 5) indicate different nephridia of the same worm. Columns 6 and 7 together describe the conditions under which the sample was collected, e.g. Serial 21, collection was made from the ascending limb of the narrow tube in the second loop, with the clamping needle at position 3 (see Fig. 1) and with the point of the pipette on the side of the needle nearer to the tip of the second loop. Column 4 gives the concentration (in terms of osmotically equivalent NaCl) of the ringer under which the worm was opened, and column 9 (Table 2 only) gives the concentration of the ringer adherent to the preparation.

taken on 7 August 1948 are also hypotonic, but less so. The samples taken from the narrow tube appear on the average to have a slightly higher concentration than the ringer.

This last result was somewhat unexpected and it seemed possible that the ringer surrounding the preparation on the slide might have become concentrated by evaporation. (It is also conceivable that the ringer might become diluted by urine leaking from the bladder.) To test this possibility six preparations were made in succession, following the normal routine exactly, and samples were taken of the ringer adhering to the preparation. These samples were found to vary from 0·75–0·8% NaCl with an average value of 0·79% NaCl as compared with 0·75% NaCl for the stock.

The preparation of the nephridium in these experiments involved the use of a binocular microscope first on a long arm over the dissecting dish, then on an ordinary stage mounting while the preparation was arranged on the slide. The slide had then to be transferred to a special stage screwed to the bench where it was viewed through a compound microscope. The micro-manipulator carrying the pipette was mounted on a large and heavy adjustable stand. It was not considered practicable to enclose all this apparatus in a moist chamber. Variations in the concentration of the ringer on the slide were therefore accepted, but in the second series of collections, after a successful collection had been made, a sample of the ringer adherent to the preparation was taken as well. Use was also made of a less concentrated ringer in the dissecting dish. The results of the first series had shown that the urine in the bladder and in the distal segment was strongly hypotonic to the ringer and it was not considered necessary to repeat these observations. In the second series attention was restricted to the narrow tube, the middle tube and the ampulla.

The results of the second series are given in Table 2. In two cases they appear to be anomalous: Serial 2, in which the adherent ringer is abnormally concentrated, and Serial 9, in which the sample collected from the middle tube shows a value nearly double that of the other samples. Neglecting these two cases the average values work out as follows:

	Urine (% NaCl)	Adherent ringer (% NaCl)
Narrow tube	0·51	0·54
Middle tube	0·51	0·56
Ampulla	0·33	0·52

In order to put all these results on a comparable basis the value for adherent ringer in each case has been equated to 100 and the value for the urine has been scaled up in proportion; a value of 0·79% NaCl has been assumed for the adherent ringer in the first series. Treated in this way all the data are presented graphically in Fig. 3.

It appears reasonable to conclude that an active process producing hypotonic urine is certainly at work in the ampulla, is possibly at work in the middle tube and

Table 2

Serial no. (1)	Date (2)	Worm (3)	Dissection ringer (% NaCl) (4)	Nephridium (5)	Part of canal (6)	Position of puncture (7)	Sample (% NaCl) (8)	Adherent ringer (% NaCl) (9)
1	11. vii. 48	*A*	0·75	(*a*)	Middle	2, base	0·52	0·71
2				(*b*)	,,	3 ,,	0·50	1·14
3				(*c*)	,,	3 ,,	0·82	0·74
4	12. vii. 48	*A*	0·75	(*a*)	Middle	1, base	0·68	0·70
5*				(*b*)	,,	1 ,,	0·42	0·41
6	13. vii. 48	*A*	0·45	(*a*)	Middle	2, base	0·49	0·49
7				(*b*)	,,	3 ,,	0·36	0·49
8		*B*		(*a*)	,,	3 ,,	0·41	0·49
9		*C*		(*a*)	,,	3 ,,	0·72	0·47
10		*D*		(*a*)	,,	0, base	0·49	0·51
11		*E*		(*a*)	,,	3 ,,	0·42	0·52
12	14. vii. 48	*A*	0·45	(*a*)	Ampulla	0, tip	0·29	0·51
13				(*b*)	,,	0 ,,	0·31	0·52
14				(*c*)	,,	0 ,,	0·29	0·49
15		*B*		(*a*)	,,	0 ,,	0·33	0·51
16				(*b*)	,,	0 ,,	0·32	0·52
17				(*c*)	,,	0 ,,	0·47	0·55
18	15. vii. 48	*A*	0·45	(*a*)	Narrow, asc.	2, tip	0·51	0·56
19				(*b*)	,, ,,	2 ,,	0·51	0·50
20		*B*		(*a*)	Narrow, des.	2 ,,	0·52	0·54
21		*C*		(*a*)	Narrow, asc.	1, tip	0·52	0·56

See legend to Table 1.
* In this experiment the ringer was diluted on the slide.

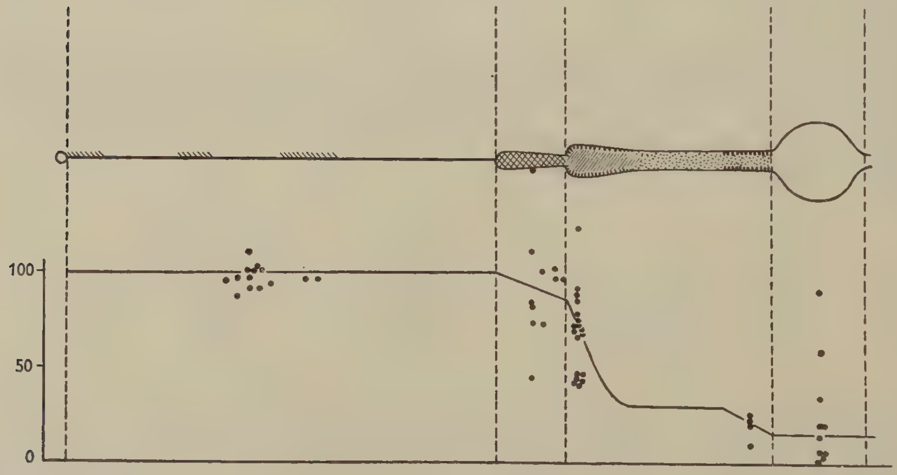

Fig. 3. To show the osmotic pressure of the urine at different levels in the nephridium. Conventions as in Fig. 1. The osmotic pressure of the ringer surrounding the nephridium has been equated to 100. Individual observations are shown as points; the line drawn through the points represents the interpretation placed upon them.

is probably not at work in the narrow tube. The results do not indicate whether the formation of hypotonic urine is due to the addition of water or to the resorption of osmotically active substances from the urine.

IV. DISCUSSION

With one possible exception, Serial 19 of Table 1, all the samples of urine collected from the ampulla or beyond it have proved to be hypotonic to the medium surrounding the nephridium, and the average value of osmotic pressure is unmistakably lower in the bladder than in the ampulla. The conclusion appears to be well-founded that a large part of the osmotic work producing hypotonic urine is carried out in the wide tube. It cannot be claimed with certainty that hypotonic urine is not formed proximal to the ampulla. Although a difference in concentration having an average value equivalent to 0·03 % NaCl has been shown to exist between the ringer and the urine in the narrow tube, this figure is based on only four observations in which a direct comparison was made. Other evidence speaks in favour of the view that there is, in fact, no significant difference in concentration. When the ringer bathing a preparation is diluted, the narrow tube immediately—in a matter of 10 sec.—increases in diameter; the middle tube usually increases in diameter also, but this takes some minutes to become apparent, and a change in the diameter of the ampulla is not noticeable until much later, if it occurs at all. This observation suggests that the wall of the narrow tube is relatively freely permeable to water and therefore implies that the urine contained in it has the same osmotic pressure as the surrounding medium. The position in regard to the middle tube is less satisfactory. Even if Serials 2 and 9 (Table 2) are neglected the scatter of the remaining observations is still considerable. It has been shown that this part of the canal is capable of taking up colloidal substances (Cordier, 1933, 1934) and it would be rash to deny the possibility of its taking up osmotically active substances as well.

It is a fair criticism that these points might have been settled if more observations had been made; but there are good reasons for believing that an extension of the work along the present lines would be unprofitable at the moment.

(*a*) Measurements of the osmotic pressure and chloride content of the coelomic fluid (Ramsay, 1949 *a*) indicate that NaCl accounts for about 50 % of the total osmotic pressure, assuming that sodium and chloride ions form a large proportion of the inorganic substances present. Other osmotically active substances, probably organic, must be responsible for the other 50 %. A similar state of affairs is found in the haemolymph of mosquito larvae (Wigglesworth, 1938; Beadle, 1939). Measurements of the osmotic pressure and chloride content of the urine indicate that the non-chloride fraction of the urine is considerably reduced as compared with that of the coelomic fluid. This reduction may conceivably be brought about by resorption of organic substances in parts of the canal proximal to the ampulla, and if this is the case it will not show itself in experiments in which organic-free frog ringer is used. This possibility cannot be tested until an analysis of the coelomic fluid has been made and an artificial medium can be prepared which is a better substitute for the coelomic fluid than is frog ringer.

(*b*) The method used for the collection of samples necessitated the interruption of the normal flow on the nephridial canal. The urine may have remained longer in the various sections of the canal and the process of forming hypotonic urine may have proceeded further than it would do under normal conditions. It is proper to point out that we know extraordinarily little of the factors which control the rate of flow. That the osmotic pressure of the medium is one of these factors is indicated by the following observation. A number of worms were allowed to remain in frog ringer equivalent to 1·4% NaCl for one week and were then opened under this same medium. The nephridial bladders, almost without exception, were found to be empty, and when the nephridia were excised and examined it was seen that the whole canal was somewhat shrunken in appearance and that ciliary activity was practically at a standstill. When the medium was replaced with frog ringer equivalent to 0·45% NaCl the narrow tube and later the middle tube opened up more fully and the cilia throughout the organ began beating. When portions of the opened worm were transferred to a dish containing the more dilute ringer the filling of the bladders became apparent in about half an hour, and after one hour some of the bladders appeared to be fully distended. The bladders of the control remained contracted.

(*c*) The method of collection also involved the cutting of the blood-vessels to the nephridium. It is obviously desirable, if technically practicable, to observe the flow of urine and to collect samples with the nephridium *in situ* and with its blood supply intact.

For these and other reasons the results reported in this paper are claimed to show no more than that ability to form hypotonic urine is associated with the wide tube of the nephridium and perhaps with the middle tube as well. The possibilities of improving technique have to be explored and a considerable amount of preliminary work has to be done before the present line of attack can be renewed with reasonable hope of advance.

The analogy between the various sections of the nephridial canal and the various sections of the vertebrate nephron, pointed out by Cordier (1934), is worthy of further consideration. In collaboration with Gérard, Cordier studied the uptake of colloidal particles of known size in amphibian nephrons with open nephrostomes (Gérard & Cordier, 1934). When injected into the body these substances accumulate in the wall of the proximal convoluted tubule, the finer particles being taken up at the proximal end and the larger particles at the distal end. Cordier repeated these experiments on *Lumbricus* and found that the finer particles were taken up at the proximal end of the middle tube and the larger particles about the middle of the middle tube. In comparing the amphibian nephron with the oligochaet nephridium he very naturally laid stress upon the similarity of function between the proximal convoluted tubule of the nephron and the middle tube of the nephridium, and he regarded the whole of the wide tube of the nephridium as representing the distal convoluted tubule of the nephron. On the basis of this analogy Cordier ventured to predict that a process of water-resorption would be found to occur in the wide tube, such as had been shown to occur in the distal convoluted tubule. This prediction was made at a time when the functions of the different parts of the amphibian nephron were still uncertain; but with the completion of the work of Richards and his associates (Richards, 1938) it became clear that the distal convoluted tubule was the site not only of water-resorption but also of salt-resorption—in fact, that it was the site of formation of hypotonic urine. The present work, although it does not confirm or refute Cordier's prediction about water-resorption, has lent support to his conception of the wide tube as the analogue of the distal convoluted tubule.

V. SUMMARY

1. Methods for the collection of samples of urine from different parts of the nephridium of *Lumbricus* are described.

2. The osmotic pressures of these samples have been measured by determination of freezing-point depression and have been compared with the osmotic pressure of the medium surrounding the nephridium.

3. The results of this comparison indicate that the ability to form hypotonic urine is certainly present in the wide tube, is possibly present in the middle tube and is probably not present in the narrow tube.

4. The analogy between the nephridium and the vertebrate nephron is discussed.

REFERENCES

Bahl, K. N. (1947). *Biol. Rev.* **22**, 109.
Beadle, L. C. (1939). *J. Exp. Biol.* **16**, 346.
Benham, W. B. (1891). *Quart. J. Micr. Soc.* **32**, 293.
Cordier, R. (1933). *C.R. Soc. Biol., Paris*, **113**, 906.
Cordier, R. (1934). *Arch. Biol., Paris*, **45**, 431.
Cuenot, L. (1898). *Arch. Biol., Paris*, **15**, 79.
Gegenbaur, C. (1853). *Z. wiss. Zool.* **4**, 221.
Gérard, P. & Cordier, R. (1934). *Biol. Rev.* **9**, 110.
Goehlich, G. (1890). *Zool. Beitr.* **2**, 133.
Goodrich, E. S. (1932). *Quart. J. Micr. Soc.* **75**, 165.
Goodrich, E. S. (1945). *Quart. J. Micr. Soc.* **86**, 113.
Kowalevsky, A. (1889). *Biol. Zbl.* **9**, 65.
Maziarski, J. (1901). *C.R. Soc. Biol., Paris*, **53**, 259.
Maziarski, J. (1905). *Poln. Arch. biol. med. Wiss.* **2**, 1.
Meisenheimer, J. (1910). *Ergebn. Zool.* **2**, 275.
Ramsay, J. A. (1946). *Nature, Lond.*, **158**, 665.
Ramsay, J. A. (1949*a*). *J. Exp. Biol.* **26**, 46.
Ramsay, J. A. (1949*b*). *J. Exp. Biol.* **26**, 57.
Richards, A. N. (1938). *Proc. Roy. Soc.* B, **126**, 398.
Rosen, F. (1911). *Z. wiss. Zool.* **98**, 135.
Schneider, G. (1896). *Z. wiss. Zool.* **61**, 363.
Wigglesworth, V. B. (1938). *J. Exp. Biol.* **15**, 235.
Willem, V. & Minné, A. (1900). *Mem. Acad. roy. Belg.* **58**, 1.

THE EFFECTS OF HIGH AND LOW TEMPERATURES ON THE EPIDERMAL MITOTIC ACTIVITY OF THE ADULT MALE MOUSE, *MUS MUSCULUS* L.

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(With Three Text-figures)

I. INTRODUCTION

The form of the diurnal cycles of epidermal mitotic activity in the adult male mouse has been shown to be determined by the times of sleeping and waking, high mitotic activity being associated with both natural and artificial sleep, and low mitotic activity with muscular exercise (Bullough, 1948*a*, *b*).

In order to define more closely the conditions which favour or depress mitosis, it is now necessary to attempt to analyse the effects of some of the factors which are known to vary between waking and sleeping. As a first step in this direction, the effects of variations in body temperature are here examined. It is well known that the body temperature is higher in waking than in sleeping, and therefore a drop in temperature may be an important prelude to active cell division. In this connexion it will be remembered that spermatogenesis is inhibited in many mammalian testes unless these organs descend into the cooler conditions of the scrotal sac.

II. MATERIAL AND METHODS

The animals used were 4-month-old males of the Kreyberg's white label and Strong's CBA strains. The two strains were mixed in approximately equal proportions in each experimental group, and no significant differences were found between them. The methods of housing and feeding were those already described by Bullough (1948*a*), and, except during the experiments, the mice were maintained at a controlled temperature of 20° C.

The study was based on the examination of pieces of ear clipped out at intervals by means of a conchotome. These earclips were fixed in Bouin's alcoholic fluid, which is also a wetting agent for the greasy skin and hair, and, after embedding, they were sectioned at a thickness of $7\,\mu$ and stained in haematoxylin and eosin.

Each earclip was about 3 mm. square, and thus contained more than enough tissue for mitosis counts to be made on ten section lengths each of 1 cm. All stages of mitosis were included in the counts. The two ears of an animal were large enough for at least 12 earclips to be taken. Each earclip was taken from that piece of ear adjacent to the hole left by the preceding clip, and as the interval was only 2 hr., there was no time for the wounding to affect the mitosis rate.

From the ten mitosis counts obtained from each earclip an average was obtained of the number of mitoses present per unit section length of 1 cm. As each experimental group consisted of ten earclips, ten averages were available from which a grand average could be calculated and from which the standard error could be derived according to the formula recommended for small samples by Simpson & Roe (1939).

The measurements of body temperature were made with a copper-constantan needle thermocouple, the needle point being inserted into the subcutaneous space of the mid-dorsal region of the back. It proved important that the mice used should be thoroughly accustomed to handling so that they could be relied upon to remain quiet while the measurement was being made. Inexperienced mice were liable to struggle while being held, and this quickly raised the body temperature. A similar difficulty related to sleeping mice which were, of necessity, awakened by the treatment, but it was found that the mere fact of wakening had little immediate effect on the body temperature provided that the animals remained quiet.

III. OBSERVATIONS

(1) *Diurnal changes in body temperature*

As a preliminary, the normal variations in body temperature were determined throughout a period of 24 hr. A group of ten mice was used, and the temperatures were taken at 2 hr. intervals starting at 10.00 hr. and continuing until 08.00 hr. on the next day. At the same times earclips were removed from which the mitosis rate was estimated, and the combined results are shown in Table 1 and Fig. 1.

Table 1. *The average subcutaneous temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten male mice throughout a period of 24 hr.*

Time of day	Subcutaneous temperatures (° C.)	Numbers of mitoses
10.00	37·8 ± 0·11	3·4 ± 0·16
12.00	35·9 ± 0·10	4·8 ± 0·21
14.00	35·2 ± 0·21	7·6 ± 0·28
16.00	35·8 ± 0·14	6·3 ± 0·25
18.00	36·9 ± 0·19	5·5 ± 0·27
20.00	37·7 ± 0·17	1·7 ± 0·11
22.00	37·7 ± 0·11	2·8 ± 0·12
24.00	37·5 ± 0·09	4·6 ± 0·20
02.00	37·4 ± 0·12	4·1 ± 0·21
04.00	37·2 ± 0·17	4·0 ± 0·17
06.00	35·4 ± 0·18	7·9 ± 0·31
08.00	35·6 ± 0·14	5·3 ± 0·29

As was to be expected, a general inverse relationship is evident between the two graphs, the low body temperature of the sleeping animal coinciding with high mitotic activity, and the high body temperature of the waking animal coinciding with low mitotic activity. It may also be remarked that the figures for mitotic activity closely parallel those previously obtained during the initial study of the diurnal cycle (Bullough, 1948*a*).

(2) *Effect of a lowered body temperature during waking*

An artificially lowered body temperature was obtained by reducing the environmental temperature, and two separate groups, each of ten male mice, were treated in this way. The animals had been reared since birth at a steady temperature of 20° C., and from this the first group was abruptly transferred to 0° C. and the second to 10° C. To test whether a lowered body temperature will induce conditions favourable to mitotic activity, the experiments were carried out during the period 16.00 to 02.00 hr. when the animals are normally active and the mitosis rate low. The body temperatures were measured and earclips were removed at 2 hr. intervals starting at 16.00 hr. when the animals were waking from their afternoon sleep. At 17.00 hr. they were transferred to the lower temperatures, and they were kept there until 23.00 hr. when they were returned to the accustomed temperature of 20° C.

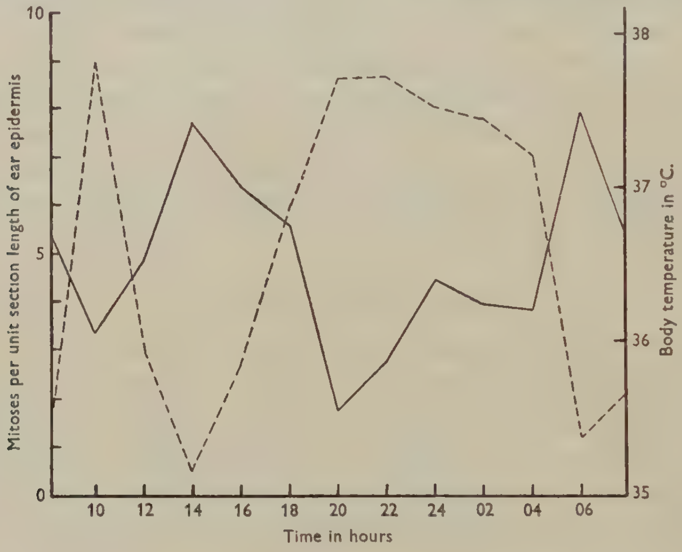

Fig. 1. The diurnal variations in the mitotic activity of the ear epidermis (continuous line) compared with the diurnal variations in the body temperature (broken line).

Table 2. *The average body temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten male mice*

Time of day	Room temperatures (° C.)	Subcutaneous temperatures (° C.)	Numbers of mitoses
16.00	20	35·5 ± 0·12	5·6 ± 0·17
18.00	0	35·7 ± 0·11	3·6 ± 0·25
20.00	0	35·4 ± 0·16	0·7 ± 0·17
22.00	0	35·3 ± 0·13	0·5 ± 0·10
24.00	20	37·9 ± 0·18	1·0 ± 0·11
02.00	20	37·6 ± 0·14	4·5 ± 0·33

The results for the first group of mice kept at 0° C. are recorded in Table 2 and Fig. 2.

The experiment was carried out at the same time as that recorded in Table 1, and from a comparison it is immediately obvious that the lowering of the body temperature was not accompanied by any rise in mitotic activity. On the contrary, it is evident that this activity actually diminished so that at 20.00 and 22.00 hr. abnormally small numbers of mitoses were found. At 20.00 hr. this depression is probably significant, while at 22.00 hr. it is certainly significant. It must be emphasized that this drop in mitotic activity was not due to any increase in muscular exercise other than shivering since, during most of the time they were in the cold, the animals were observed to be clustering together for warmth.

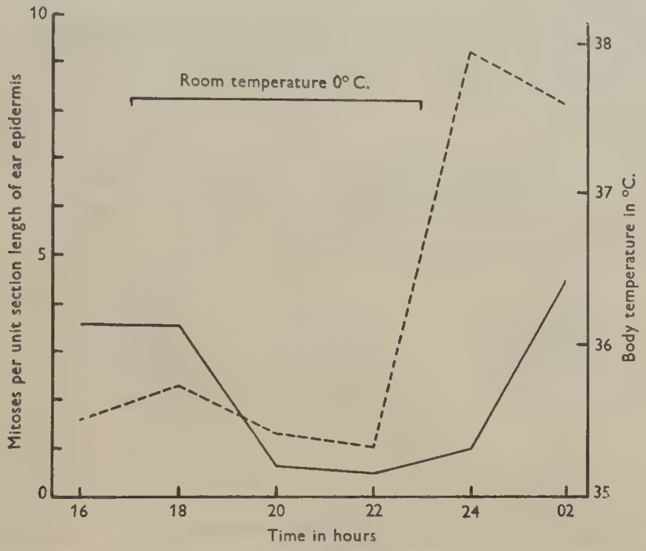

Fig. 2. The changes in the mitotic activity of the ear epidermis (continuous line) and in the body temperature (broken line) of mice exposed to a temperature of 0° C. from 17.00 to 23.00 hr.

Table 3. *The average body temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten male mice*

Time of day	Room temperatures (° C.)	Subcutaneous temperatures (° C.)	Numbers of mitoses
16.00	20	35·6 ± 0·13	5·1 ± 0·25
18.00	10	36·1 ± 0·15	4·3 ± 3·20
20.00	10	36·2 ± 0·06	1·3 ± 0·13
22.00	10	36·5 ± 0·10	2·6 ± 0·23
24.00	20	37·4 ± 0·11	1·7 ± 0·16
02.00	20	37·4 ± 0·07	3·8 ± 0·29

The second group of mice which were kept at a temperature of 10° C. from 17.00 to 23.00 hr. gave the results recorded in Table 3.

Here again the lowered environmental temperature entirely failed to induce any rise in mitotic activity, and again the tendency was in the other direction. All the mitosis counts for the low-temperature period are below those of the controls (Table 1), but the effect is not statistically significant.

(3) *Effect of a lowered body temperature during sleep*

In an attempt to obtain a stronger and more significant effect on the mitosis rate, the low-temperature experiment was repeated at a time when the animals are normally asleep with high mitotic activity. This was done between 10.00 and 20.00 hr., the ten mice being transferred to a temperature of 0° C. at 11.00 hr. and kept there until 17.00 hr. when they were returned to the usual temperature of 20° C. While it is possible that the low temperature interfered to some extent with their sleep, the animals were repeatedly observed to be resting with closed eyes and to be huddled together for warmth. The results of this experiment and of a control experiment which was carried out at the same time are given in Table 4.

Table 4. *The average body temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten control mice and of ten mice exposed to low temperature.*

Time of day	Control mice kept at 20° C.		Mice kept at 0° C. from 11.00 to 17.00 hr.	
	Body temperatures (° C.)	Numbers of mitoses	Body temperatures (° C.)	Numbers of mitoses
10.00	37·6±0·09	3·3±0·13	37·4±0·10	3·0±0·17
12.00	35·7±0·12	5·2±0·21	35·8±0·11	4·1±0·13
14.00	35·3±0·18	7·5±0·33	35·2±0·12	3·6±0·16
16.00	36·0±0·14	6·6±0·30	34·7±0·13	3·5±0·09
18.00	36·8±0·17	6·0±0·28	38·2±0·18	4·7±0·11
20.00	37·5±0·20	1·6±0·10	37·8±0·16	3·9±0·10

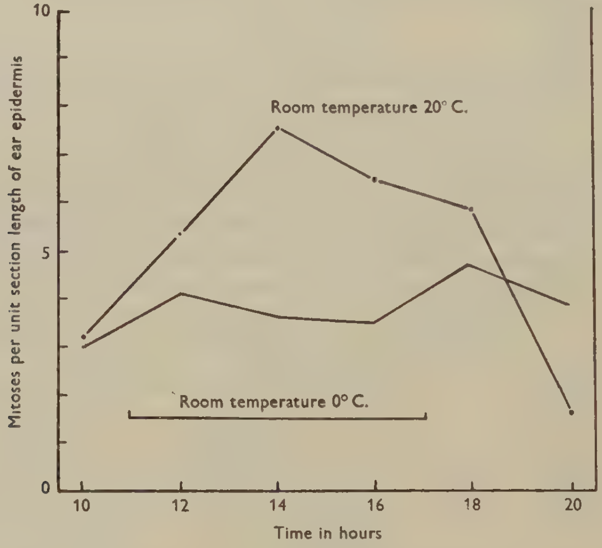

Fig. 3. The changes in the mitotic activity of the ear epidermis of control mice (above) and of mice exposed to a temperature of 0° C. from 11.00 to 17.00 hr. (below).

A comparison between these two sets of mitosis counts is made in Fig. 3, and it demonstrates beyond doubt that a reduced environmental temperature results in a reduced mitosis rate.

(4) *Effect of a raised body temperature during sleep*

These experiments were carried out in a similar manner, but with the difference that the mice were transferred to a warm chamber which raised the body temperature. Again the reactions of two groups, each of ten male mice, were examined, one group being suddenly transferred to a temperature of 30° C. and the other to 36° C. Since the experiments were designed to discover whether a raised body temperature typical of the waking animal prevents the development of a high mitosis rate, they were carried out between 10.00 and 20.00 hr. so as to include the afternoon sleep period when mitotic activity is normally high. The animals were moved to the higher temperatures at 11.00 hr., and at 17.00 hr. they were returned to the normal temperature of 20° C. Starting at 10.00 hr., measurements of the body temperatures were made at 2 hr. intervals, and at the same times earclips were taken for a study of mitotic activity. The results obtained from the mice exposed to a room temperature of 30° C. are shown in Table 5, and the controls which were examined at the same times gave the results in Table 1.

Table 5. *The average body temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten male mice*

Time of day	Room temperatures (° C.)	Subcutaneous temperatures (° C.)	Numbers of mitoses
10.00	20	37·6 ± 0·12	3·3 ± 0·31
12.00	30	36·4 ± 0·14	5·8 ± 0·44
14.00	30	36·7 ± 0·14	9·7 ± 0·32
16.00	30	36·8 ± 0·19	4·1 ± 0·23
18.00	20	37·6 ± 0·09	2·4 ± 0·19
20.00	20	37·4 ± 0·11	1·3 ± 0·12

Table 6. *The average body temperatures and the average numbers of mitoses per unit section length of ear epidermis of ten male mice*

Time of day	Room temperatures (° C.)	Subcutaneous temperatures (° C.)	Numbers of mitoses
10.00	20	37·7 ± 0·17	2·5 ± 0·19
12.00	36	38·1 ± 0·13	6·5 ± 0·36
14.00	36	38·0 ± 0·25	6·3 ± 0·44
16.00	36	37·9 ± 0·06	5·3 ± 0·26
18.00	20	35·9 ± 0·02	3·0 ± 0·15
20.00	20	36·7 ± 0·08	1·7 ± 0·17

It is evident that this treatment in no way interfered with the normal rise in mitotic activity which accompanied the afternoon sleep, and it may be added that it did not interfere with the sleep itself.

The results for the second group of mice are shown in Table 6. These animals were transferred to an environmental temperature of 36° C. which preliminary experiments had shown to be the highest they could tolerate without serious discomfort. Temperatures of 37° C. caused great distress and occasional death. These experiments were also carried out at the same time as those in Table 1.

During this experiment it was observed that the height of the room temperature interfered to some extent with the animals' rest. They went to sleep before 11.00 hr., and this was followed by a rise in mitotic activity at 12.00 hr. However, before 13.00 hr. the animals became restless, and short periods of sleep alternated with visits to the drinking bottle. It was probably this restlessness, and not the high temperature as such, which prevented any rise in the mitosis rate after 12.00 hr.

Further experiments were performed which were equally negative and which it does not appear necessary to repeat in full. They involved the use of pyretics, such as dinitrophenol, to induce a raised body temperature, and they did not result in any significant changes in the mitosis rate.

IV. CONCLUSIONS

It is evident from these experiments that an artificial reduction in the body temperature of a waking mouse to a level typical of sleep is not followed by a rise in mitotic activity, and similarly that the artificial prevention of a drop in the body temperature of a sleeping mouse does not interfere with the normal rise in mitotic activity which takes place at this time.

However, one significant result does emerge, namely that a reduction in mitotic activity occurs in mice which are kept at a low environmental temperature. This may well be related to a previous observation (Bullough, 1948*b*) that muscular exercise is accompanied by a reduction in mitotic activity. A common factor in these two situations is the drain on the sugar reserves, in the one case to maintain the body temperature, and in the other to furnish energy for muscular contraction. This aspect of the problem of diurnal mitosis cycles is examined in the following paper.

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REFERENCES

BULLOUGH, W. S. (1948*a*). *Proc. Roy. Soc.* B, **135**, 212.
BULLOUGH, W. S. (1948*b*). *Proc. Roy. Soc.* B, **135**, 233.
SIMPSON, G. G. & ROE, A. (1939). *Quantitative Zoology.* New York.

THE RELATION BETWEEN THE EPIDERMAL MITOTIC ACTIVITY AND THE BLOOD-SUGAR LEVEL IN THE ADULT MALE MOUSE, *MUS MUSCULUS* L.

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(With Ten Text-figures)

I. INTRODUCTION

The diurnal cycle of mitotic activity in the adult male mouse has been described in detail (Bullough, 1948*a*), and its relation to periods of waking and sleeping has been emphasized (Bullough, 1948*b*). Since then a search has continued for the critical factor, or factors, which are responsible for the form of the cycle, the first factor to be investigated being that of body temperature (Bullough, 1949). It has been found that the normal variations in body temperature cannot be the cause of the diurnal mitosis cycle, and that abnormal variations have little or no effect on mitotic activity. However, from this and from the previous work, two significant results have emerged. The first is that experimentally induced exercise results in an abnormally reduced mitosis rate, and the second that a similar effect is induced by extreme cold. A common factor in both these situations is the drain on the sugar reserves, in the first case for the movements of the limbs, and in the second for the production of heat. The present paper records the results of experiments which were performed to test the theory that a connexion exists between mitotic activity and blood sugar.

II. MATERIAL AND METHODS

(1) *The mice.* The animals used were of the Kreyberg's white label and Strong's CBA strains, and no differences in response were found between them. All were healthy males in full breeding condition, and all were between the ages of 3 and 6 months. They received a diet which included whole oats, dog biscuit, and rat cake soaked in cod-liver oil, and their daily food was always given them between 09.00 and 10.00 hr. It may be important to add that sufficient was always given for there to be an abundance constantly present in the cages throughout the 24 hr. The laboratory was maintained at 20° C., and was artificially lighted between 09.00 and 18.00 hr. daily. The experiments were carried out between September and January.

(2) *Estimation of mitotic activity.* The mitosis rate was measured in the ear epidermis by the earclip technique already described (Bullough, 1949). It may be repeated here that all stages of mitosis were included in the counts which were made on 1 cm. lengths of epidermis in sections cut 7μ thick, and that from each earclip

ten such counts were made and the average taken. As each experimental group consisted of earclips from ten mice, ten figures were available from which a grand mean and standard error were calculated according to the formula for small samples recommended by Simpson & Roe (1939).

(3) *Estimation of blood sugar.* To obtain samples of blood it proved necessary to kill the mice. This was done by picking them up as gently as possible, and then quickly driving a spike into the brain. Immediately afterwards the throat was cut widely open, and the heart, continuing to beat, drove the blood into a small dish. The blood was constantly stirred to discourage clotting, which otherwise was extremely rapid, and as quickly as possible (in all cases within 45 sec.) two samples, each of 0·1 c.c., were taken. The glucose content of these samples was then estimated by a modification of the Hagedorn and Jensen technique. Thus for each group of ten mice, twenty blood-sugar estimations were made, and from these figures the averages and standard errors were calculated.

III. OBSERVATIONS

(1) *Preliminary experiments*

A series of preliminary experiments was performed in an attempt to establish some connexion between mitotic activity and the blood-sugar level. Repeated attempts were made by means of injections of glucose to obtain a stimulation of mitosis in waking mice, in which such activity is normally low. Following the evidence of Bissinger, Lesser & Zipf (1923) that the carbohydrate content of a 20 g. mouse after an 18 hr. fast is approximately 20 mg. of glycogen and 20 mg. of glucose, quantities ranging from 10 to 40 mg. of glucose in isotonic water solution were given in single or in repeated subcutaneous injections. Later, starch was substituted in order that the release of glucose into the blood should be considerably slowed, and that, in consequence, its effects should be felt over a longer period.

In these experiments the time of injection was 09.00 hr. (1 hr. before the time of minimum mitotic activity), and 20 mg. of starch in 0·9% saline was found to be a suitable dose. The response to this treatment was immediate, the mitosis rate rising to a high level at a time when it would normally be low.

(2) *The diurnal cycles*

Encouraged by this result, a comparison was next made between the normal cyclic variations in the blood-sugar level and in the mitosis rate throughout the 24 hr. Groups of ten mice each were killed at 2 hr. intervals starting at 10.00 hr. on one day and ending at 08.00 hr. on the next. From each mouse were taken two blood samples and a piece of ear, and the results are recorded in Table 1 and Fig. 1.

Contrary to what might have been expected from the preliminary experiments, the one graph is the inverse of the other. At times of high mitotic activity the blood-sugar level is low, while at times of low mitotic activity the blood-sugar level is high. This, of course, is related to periods of waking and sleeping (Bullough, 1948*a*). Mitotic activity is high and the blood-sugar level is low during sleep, while mitotic activity is low and the blood-sugar level is high during the hours of waking.

If, as was suggested by the preliminary experiments, high mitotic activity is dependent on an abundance of blood sugar, it becomes necessary to attempt to define as clearly as possible what is meant by an abundance. It may be that during sleep when the blood-sugar level is low there is actually an abundance, since the demand of the muscles is at a minimum, while during waking when the sugar level is high the demand of the muscles is such that no abundance exists. To test this theory the following experiments were performed.

Table 1. *The diurnal cycles in the blood-sugar level and in the mitotic activity of the ear epidermis as shown by groups of ten male mice killed at 2 hr. intervals*

Time of day	Average blood-sugar levels (mg./100 c.c.)	Average numbers of mitoses per unit section length
10.00	165·4 ± 3·26	2·5 ± 0·16
12.00	156·3 ± 4·43	5·6 ± 0·37
14.00	149·3 ± 3·06	9·8 ± 0·47
16.00	155·3 ± 5·14	5·3 ± 0·26
18.00	162·5 ± 4·87	2·8 ± 0·22
20.00	172·0 ± 3·57	1·4 ± 0·15
22.00	155·5 ± 3·85	4·0 ± 0·27
24.00	145·8 ± 5·30	5·8 ± 0·20
02.00	139·0 ± 5·18	10·1 ± 0·37
04.00	141·9 ± 3·10	9·4 ± 0·30
06.00	142·5 ± 3·37	7·9 ± 0·39
08.00	147·9 ± 5·74	3·6 ± 0·29

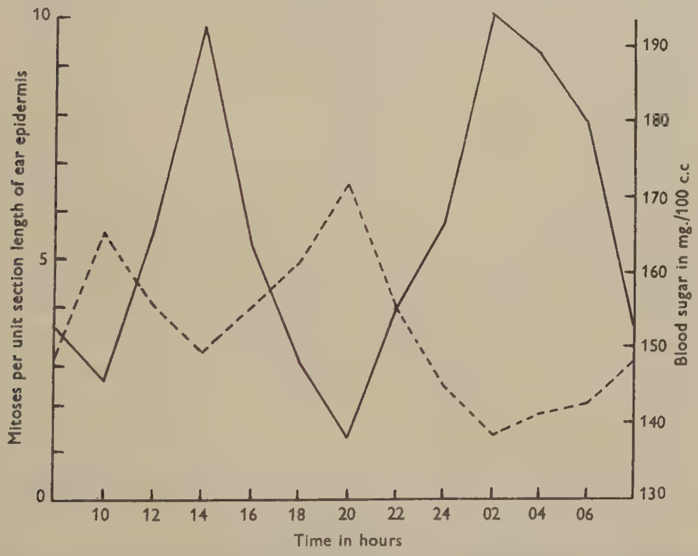

Fig. 1. The diurnal variations in the mitotic activity of the ear epidermis (continuous line) compared with the diurnal variations in the blood-sugar concentration (broken line).

(3) *The effect of starch injections*

It seemed possible that if the already high blood-sugar level typical of the waking animal could be raised still higher it might result in the raising of the mitosis rate to a level typical of sleep while the animals were still awake and active. Indeed it seemed probable that this was what had actually happened in the preliminary experiments. It was decided to try to raise the mitosis rate to a maximum at 10.00 hr. because at this time, when the rate is normally at a minimum, the result would have a maximum significance. The animals could always be relied upon to be awake and active at 10.00 hr. since they were accustomed to being fed just before this time.

Table 2. *The effects of an injection of 20 mg. of starch at 08.30 hr. on the blood-sugar level and on the epidermal mitosis rate of groups of ten adult male mice*

Time of day	Average blood-sugar levels (mg./100 c.c.)	Average numbers of mitoses per unit section length
08.00	150·1 ± 4·33	3·8 ± 0·27
09.00	191·4 ± 5·30	5·5 ± 0·32
10.00	209·9 ± 4·41	10·1 ± 0·30
12.00	146·6 ± 5·42	10·4 ± 0·46
14.00	130·1 ± 2·62	9·3 ± 0·31

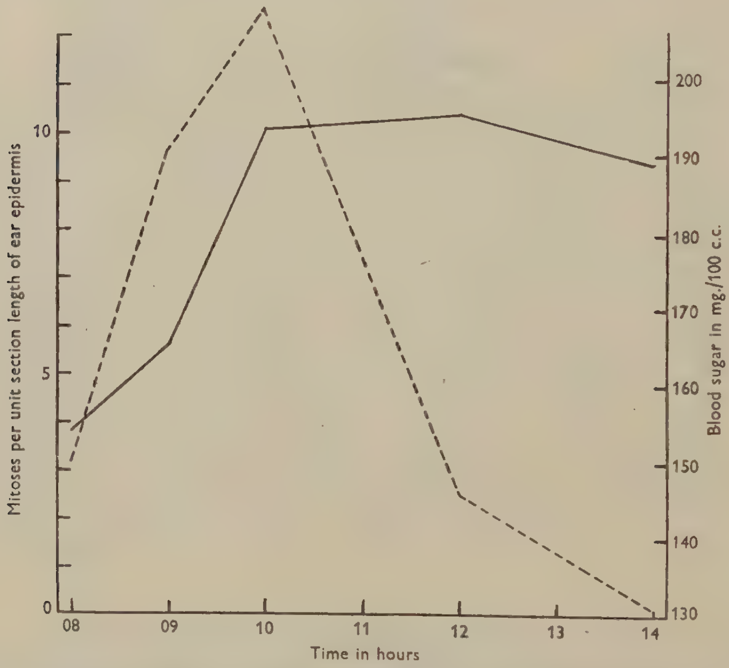

Fig. 2. The changes in the mitotic activity of the ear epidermis (continuous line) and in the blood-sugar concentration (broken line) following the subcutaneous injection of 20 mg. of starch at 08.30 hr.

A series of experiments, elaborations of the preliminary experiments recorded above, was carried out with this intention. In the first, groups of ten Strong's CBA mice were killed at 08.00, 09.00, 10.00, 12.00 and 14.00 hr., and from each animal there were taken two blood samples and a piece of ear. At 08.30 hr. the mice then alive were each injected subcutaneously with 0·5 c.c. of isotonic saline containing 20 mg. of starch. The results obtained are given in Table 2 and Fig. 2.

It is evident that as the blood-sugar level rose steeply to reach a maximum after about 1½ hr. the mitosis rate also rose until at 10.00 hr. a degree of mitotic activity similar to that normally found in sleeping mice was obtained in mice which were awake and active. By 12.00 hr. the blood-sugar level had fallen, but by this time the mice had started their mid-day sleep and the fall was not accompanied by a reduction in mitosis. By 14.00 hr. the blood sugar was markedly subnormal, apparently as an over-compensation for the earlier excess, but with the animals still sound asleep, the mitosis rate remained high.

In a second confirmatory experiment blood-sugar estimations were not made, and the mice were kept alive. Two groups each of ten Strong's CBA mice were used, and from each animal an earclip was removed at 08.00 hr. and at hourly intervals thereafter until 14.00 hr. At 08.30 hr. the mice in the control group each received an injection of 0·5 c.c. of isotonic saline, while the mice in the experimental group each received 0·5 c.c. of isotonic saline containing 20 mg. of starch. From the earclips the changes in mitotic activity were assessed, and the results are given in Table 3.

Table 3. *The changes in the epidermal mitotic activity of ten control mice and of ten mice injected with 20 mg. of starch at 08.30 hr.*

Time of day	Average numbers of mitoses per unit section length	
	In control mice injected with saline	In mice injected with saline and starch
08.00	6·4±0·34	5·6±0·25
09.00	2·8±0·22	6·2±0·29
10.00	1.2±0·12	6·3±0·29
11.00	2·0±0·11	4·2±0·30
12.00	3·7±0·21	6·8±0·37
13.00	6·5±0·45	11·8±0·42
14.00	9·0±0·41	12·8±0·47

In these results the stimulation achieved at 10.00 hr. was not so great, but it was sufficiently striking for it to be said that the two experiments are confirmatory. It is also possible to draw a further conclusion from this experiment, for it is evident that, with the approach of the early afternoon rest period, the injection of 20 mg. of starch induces an unusually rapid rise in mitotic activity to the high levels seen at 13.00 and 14.00 hr. As a further check on this effect, the experiment was repeated once again, and this time the earclips were taken hourly until 15.00 hr. The results are given in Table 4 and Fig. 3.

Table 4. *The changes in the epidermal mitotic activity of ten control mice and of ten mice injected with 20 mg. of starch at 08.30 hr.*

Time of day	Average numbers of mitoses per unit section length	
	In control mice injected with saline	In mice injected with saline and starch
08.00	6·0±0·32	6·4±0·27
09.00	3·9±0·19	6·3±0·29
10.00	1·7±0·11	3·6±0·16
11.00	1·9±0·15	4·2±0·21
12.00	3·8±0·20	9·9±0·33
13.00	5·5±0·36	9·2±0·29
14.00	7·1±0·30	10·6±0·41
15.00	5·9±0·27	7·2±0·32

In this case the stimulation achieved at 10.00 hr. was even less than before, but again the starch injection induced a great increase in the rate of mitotic activity at the beginning of the rest period. By 12.00 hr. the mitotic activity in the starch-injected animals had reached an abnormally high level which was maintained until after 14.00 hr.

The absence in this experiment of any great mitosis stimulation at 10.00 hr. was unexpected, and prompted two further repetitions of the experiment. In one of these only slight mitosis stimulation was obtained at 10.00 hr., but in the other the stimulation was great. In both the rate of increase of mitotic activity, as well as the level reached at the time of the mid-day sleep, was significantly higher after the injection of starch. Thus, in spite of some uncertainty concerning the time lag, it may safely be concluded that a rise in the blood-sugar level, as induced by the injection of 20 mg. of starch, is followed by a rise in mitotic activity. In the experiments which involved the shortest time lags, this stimulation was obtained in active waking animals so that their mitotic activity approximated to that normally found during sleep.

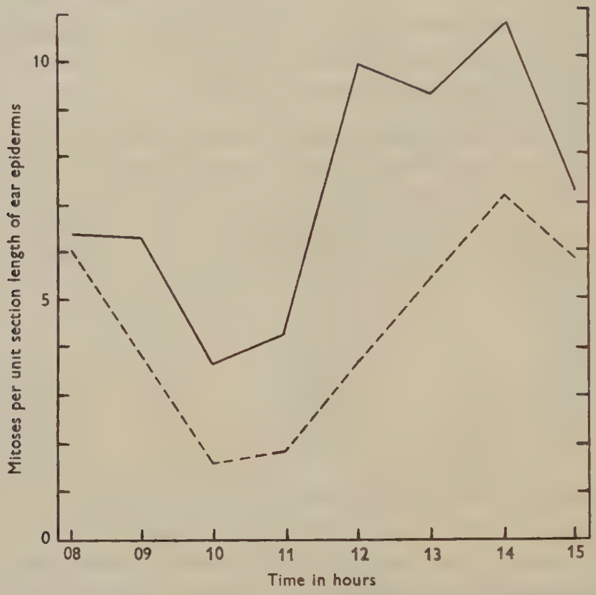

Fig. 3. The changes in the mitotic activity of the ear epidermis of mice injected at 08.30 hr. with 20 mg. of starch dissolved in saline (continuous line) and with saline alone (broken line).

(4) *The effects of various doses of starch*

The question now arises as to what limit exists to the stimulation which can be achieved in this way. To answer this, six groups, each of ten male mice, were injected either with isotonic saline as controls, or with from 10 to 50 mg. of starch. The injections, all subcutaneous, were made at 08.30 hr., and earclips were taken hourly from 08.00 to 14.00 hr. The control mice each received 1 c.c. of isotonic saline, while the experimental mice each received either 10, 20, 30, 40, or 50 mg.

of starch dissolved in not more than 1 c.c. of isotonic saline. The effects on the mitotic activity of the ear epidermis induced by these various treatments are shown in Table 5.

Table 5. *The changes in epidermal mitotic activity in groups of ten mice following injections of various quantities of starch at* 08.30 *hr.*

Time of day	Average numbers of mitoses per unit section length					
	In control mice injected with saline	In experimental mice injected with various quantities of starch				
		10 mg.	20 mg.	30 mg.	40 mg.	50 mg.
08.00	7·4±0·31	6·6±0·25	6·2±0·29	6·9±0·30	6·5±0·31	7·2±0·26
09.00	2·1±0·12	6·8±0·32	6·3±0·33	7·3±0·29	4·8±0·23	1·8±0·19
10.00	1·7±0·08	6·2±0·30	7·1±0·31	4·0±0·11	1·5±0·14	1·0±0·09
11.00	2·0±0·15	3·1±0·18	7·0±0·19	3·1±0·14	1·8±0·12	0·8±0·10
12·00	3·7±0·21	7·3±0·29	8·7±0·26	6·4±0·31	5·2±0·26	4·1±0·19
13.00	8·8±0·39	12·1±0·36	9·9±0·31	8·6±0·32	5·7±0·21	3·9±0·17
14.00	8·1±0·40	12·0±0·38	11·4±0·39	10·5±0·35	7·6±0·36	4·2±0·20

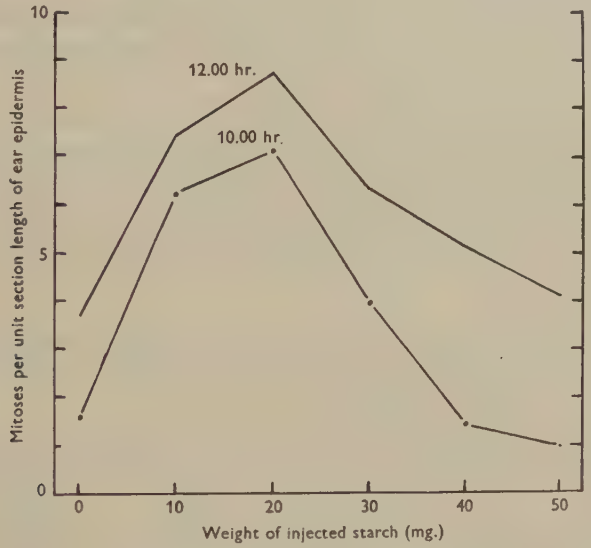

Fig. 4. Graphs illustrating the different degrees of stimulation of the mitotic activity of the ear epidermis at 10.00 and 12.00 hr. after injections of from 0 to 50 mg. of starch at 08.30 hr.

A comparison with Table 1 shows that the injections of saline alone had little or no effect on mitotic activity. Some stimulation of mitosis was evidently induced by the injections of 10 mg. and of 30 mg. of starch at all times after 09.00 hr., but this was not so considerable as that induced by 20 mg. With 40 mg. the result was a marked depression of activity, and with 50 mg. the depression was so acute that it seriously interfered with the rise in the mitosis rate which normally occurs between 12.00 and 14.00 hr., although it did not stop the animals from sleeping at this time.

One method of illustrating these results is that in Fig. 4, which compares the levels of mitotic activity reached at 10.00 hr. and again at 12.00 hr. This shows clearly the limitations to the stimulation of mitosis by means of starch injections, the maximum reaction being developed after treatment with about 20 mg.

(5) *The effect of blood phosphate*

The results so far obtained support the conclusion that, within limits, abnormal increases in the blood-sugar level result in abnormal increases in epidermal mitotic activity. However, the enigma remains that in the normal animal a relatively high concentration of blood sugar is associated with a low rate of cell division during waking, while a relatively low concentration of blood sugar is associated with a high rate of cell division during sleeping. Important as the sugar concentration may be, it appears certain that other more important factors are acting to limit mitosis, and to determine the form of the diurnal cycle. At this stage of the investigation it was decided, in discussion with Prof. J. B. S. Haldane, F.R.S., that an examination of the effect of the blood-phosphate level might prove valuable. One of the interesting points about the normal blood-phosphate concentration is that it is higher during sleep than during waking, and in this it resembles mitotic activity. In theory, therefore, it could be an important factor in the control of mitosis, and also, since phosphates are so closely involved with sugar in the release of energy, a link with the foregoing experiments is possible.

In these experiments sixty mice were studied, ten being injected with 1 c.c. of normal saline, ten with 20 mg. of starch alone, ten with 20 mg. of starch and 10 mg. of Na_2HPO_4, $2H_2O$, and ten each with 5, 10 and 15 mg. of Na_2HPO_4, $2H_2O$ alone. The starch solution was made up as before in normal saline, while the phosphate was made up as an N/10 solution in distilled water with the pH adjusted to 7·4. The results obtained are shown in Table 6 and in Fig. 5.

Table 6. *The changes in epidermal mitotic activity in groups of ten male mice following injections at* 08.30 *hr. of various quantities of* Na_2HPO_4, $2H_2O$ *with and without the addition of* 20 *mg. of starch*

Time of day	Average numbers of mitoses per unit section length in mice injected with					
	Saline alone	20 mg. starch	20 mg. starch and 10 mg. Na_2HPO_4	5 mg. Na_2HPO_4 alone	10 mg. Na_2HPO_4 alone	15 mg. Na_2HPO_4 alone
08.00	6·6 ± 0·19	7·0 ± 0·11	6·7 ± 0·12	6·1 ± 0·10	6·0 ± 0·18	6·4 ± 0·11
09.00	5·1 ± 0·18	6·1 ± 0·11	4·9 ± 0·10	2·8 ± 0·10	3·5 ± 0·08	4·1 ± 0·10
10.00	2·7 ± 0·06	3·3 ± 0·08	3·1 ± 0·09	2·6 ± 0·06	2·2 ± 0·10	2·7 ± 0·08
11.00	1·3 ± 0·05	6·1 ± 0·19	8·5 ± 0·14	1·4 ± 0·05	1·1 ± 0·05	4·9 ± 0·06
12.00	4·0 ± 0·10	9·4 ± 0·14	12·6 ± 0·21	2·9 ± 0·09	5·2 ± 0·07	6·0 ± 0·17
13.00	7·3 ± 0·15	10·5 ± 0·12	12·8 ± 0·25	7·1 ± 0·11	8·4 ± 0·12	9·6 ± 0·19
14.00	7·0 ± 0·14	9·5 ± 0·16	10·8 ± 0·18	7·1 ± 0·10	6·4 ± 0·09	6·7 ± 0·12

Again the mice injected with saline alone showed no response to the treatment, while the mice injected with 20 mg. of starch showed an increased rate of mitotic activity from 11.00 hr. onwards. However, this response to starch alone was considerably exceeded by those mice which received 10 mg. of phosphate in addition to 20 mg. of starch. In them the rate of increase of the mitotic activity and the level reached were consistently higher. No such response was developed in any of the mice which received the phosphate alone, although it is possible that those which received 15 mg. did experience some degree of stimulation.

It follows from these results that while a rise in the blood-phosphate level may strengthen the reaction produced by starch alone, it is not by itself capable of inducing an abnormal stimulation of mitotic activity, and it cannot be considered as the primary factor responsible for the rise in this activity which normally takes place during sleep.

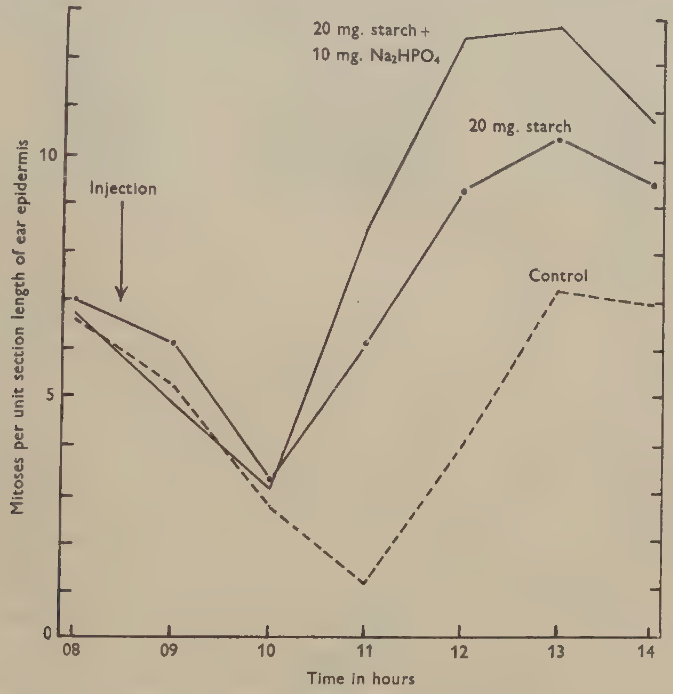

Fig. 5. The changes in the mitotic activity of the ear epidermis in control mice, in mice injected with 20 mg. of starch, and in mice injected with 20 mg. of starch together with 10 mg. of Na_2HPO_4, $2H_2O$.

(6) *The effects of insulin*

Since the above experiments indicated that some connexion exists between blood sugar and mitotic activity, it appeared of interest to discover what effects are exerted on the rate of cell division by substances which reduce the availability of sugar. The first substance tested in this way was insulin, and after preliminary experiments designed to discover the tolerance of the animals to various doses, injections were given to mice which were beginning their mid-day rest. It was found that the strongest effects were produced by two doses, each of $\frac{1}{50}$ unit dissolved in 0·5 c.c. of isotonic saline, given at 12.30 and 13.30 hr. This was almost the greatest amount of insulin that could be given without causing distress and without interfering with the normal observance of the mid-day sleep period. In the first experiment the mice were killed hourly in groups of ten animals each from 10.00 to

16.00 hr., and estimations were made of the blood-sugar level and of the rate of epidermal mitotic activity in the ear. The results were striking, and are given in Table 7 and Fig. 6.

Table 7. *The effects of two injections of* $\frac{1}{50}$ *unit of insulin at* 12.30 *and* 13.30 *hr. on the blood-sugar level and on the epidermal mitosis rate of groups of ten adult male mice.*

Time of day	Average blood-sugar levels (mg./100 c.c.)	Average numbers of mitoses per unit section length
10.00	165·4 ± 2·95	2·9 ± 0·18
11.00	166·4 ± 3·16	2·1 ± 0·21
12.00	161·2 ± 3·50	3·9 ± 0·15
13.00	139·6 ± 4·23	2·5 ± 0·17
14.00	138·6 ± 3·83	1·9 ± 0·08
15·00	186·1 ± 4·11	0·3 ± 0·10
16.00	201·2 ± 3·99	1·7 ± 0·19

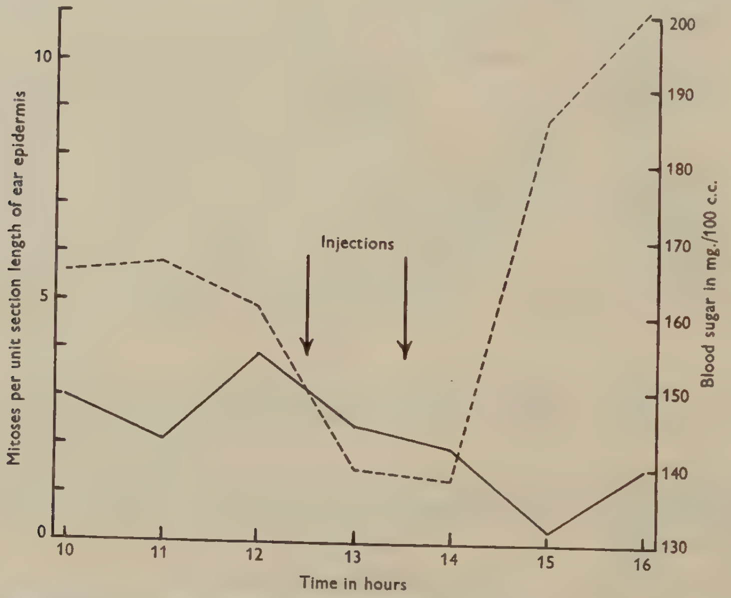

Fig. 6. The changes in the mitotic activity of the ear epidermis (continuous line) and in the blood-sugar concentration (broken line) after injections, each of $\frac{1}{50}$ unit of insulin, at 12.30 and 13.30 hr.

A sharp fall in the blood-sugar level ½ hr. after the first injection was accompanied by a reduction in mitotic activity. This reduction became more acute after the second injection, and an extraordinary low level was reached by 15.00 hr. so that the normal early afternoon peak of mitotic activity was eliminated altogether. About 15.00 hr. the blood-sugar level rose rapidly, apparently due to an over-compensation, and this was followed by a slight rise in the mitosis rate.

The experiment was then repeated without killing the mice involved, and earclips were taken hourly from 10.00 to 16.00 hr. The control and experimental groups each contained ten adult male mice. At each injection the former were given 0·5 c.c. of isotonic saline, and the latter 0·5 c.c. of isotonic saline containing $\frac{1}{50}$ unit of insulin. The results are recorded in Table 8 and in Fig. 7.

Table 8. *The changes in the epidermal mitotic activity of ten control mice and of ten mice injected with* $\frac{1}{50}$ *unit of insulin at* 12.30 *and* 13.30 *hr.*

Time of day	Average numbers of mitoses per unit section length	
	In control mice injected with saline	In mice injected with saline and insulin
10.00	2·2±0·16	3·1±0·21
11.00	3·0±0·22	3·6±0·19
12.00	4·1±0·17	4·9±0·12
13.00	5·8±0·27	2·6±0·18
14.00	7·3±0·21	1·5±0·16
15.00	5·8±0·31	0·1±0·05
16·00	4·2±0·15	1·4±0·19

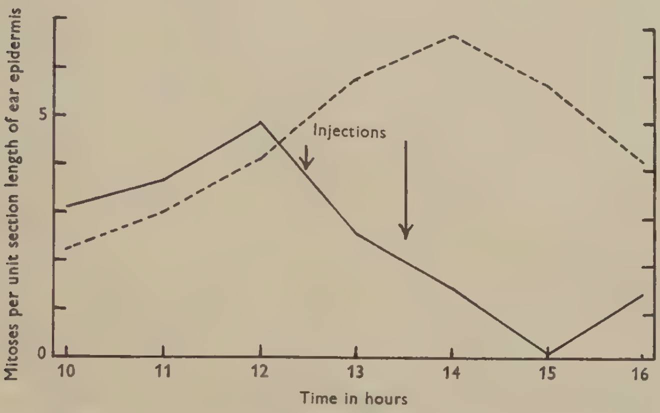

Fig. 7. The changes in the mitotic activity of the ear epidermis of mice injected at 12.30 and 13.30 hr. with $\frac{1}{50}$ unit of insulin (continuous line) as compared with controls (broken line).

Full confirmation of the results of the first experiment is demonstrated, and indeed by 15.00 hr. mitotic activity was almost totally eliminated.

(7) *The effects of phloridzin*

Experiments involving the use of phloridzin were next carried out. This substance, while not at first causing any reduction in the blood-sugar level, impedes phosphorylation, and so opens the kidneys for the escape of sugar with the urine. After preliminary experiments, a dose of 3 mg. of phloridzin dissolved in 0·5 c.c. of isotonic saline and injected at 12.00 hr. was established as suitable. In the first experiment, groups of ten mice each were killed at hourly intervals from 10.00 to 16.00 hr., and determinations were made of the blood-sugar levels and of the epidermal mitosis rates. The results are given in Table 9 and Fig. 8.

The injection of phloridzin was followed by a rise in the concentration of the blood sugar, and by a fall in the mitosis rate. The effect obtained is longer and steadier than that produced by insulin, and the mitosis rate remained low at 16.00 hr. when the last clip was taken. It should be added that the mice observed the normal mid-day rest period in an entirely normal manner.

Table 9. *The effects of an injection of 3 mg. of phloridzin at* 12.00 *hr. on the blood-sugar level and on the epidermal mitosis rate of groups of ten adult male mice*

Time of day	Average blood-sugar levels (mg./100 c.c.)	Average numbers of mitoses per unit length section
10.00	162·1 ± 3·51	2·9 ± 0·14
11.00	161·7 ± 3·67	3·3 ± 0·23
12.00	155·1 ± 4·03	3·6 ± 0·21
13.00	156·9 ± 3·77	2·0 ± 0·13
14·00	197·3 ± 4·63	1·7 ± 0·15
15·00	198·6 ± 4·75	1·7 ± 0·23
16.00	199·4 ± 5·11	2·1 ± 0·20

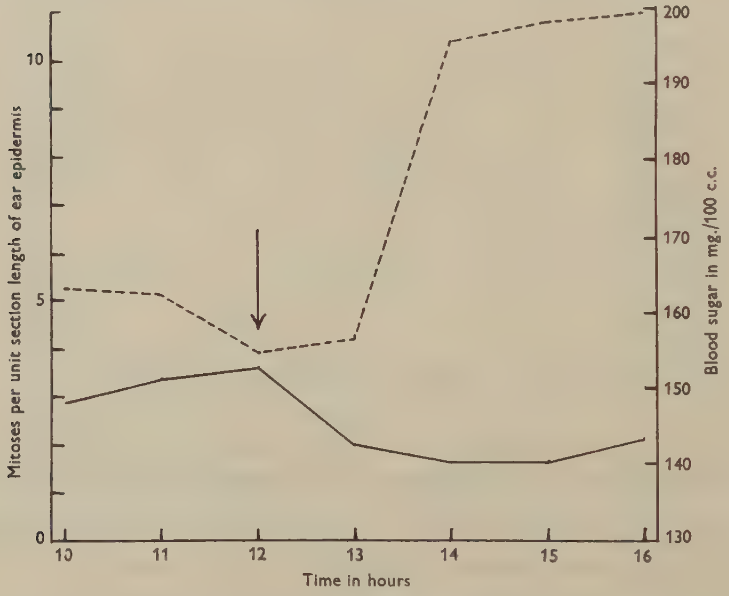

Fig. 8. The changes in the mitotic activity of the ear epidermis (continuous line) and in the blood-sugar concentration (broken line) after an injection of 3 mg. of phloridzin at 12.00 hr.

The experiment was then repeated without killing the mice involved, ten control males being injected at 12.00 hr. with 0·5 c.c. of isotonic saline, and ten mice with 3 mg. of phloridzin in 0·5 c.c. of isotonic saline. Earclips were taken at hourly intervals from 10.00 to 16.00 hr., and the results are shown in Table 10 and Fig. 9.

The depression of mitosis observed in the experimental animals is similar in timing and degree to that in the first phloridzin experiment.

It can be concluded that both insulin and phloridzin have a powerful depressing effect on the mitosis rate. The effect of the former is short-lived but acute, while that of the latter is more sustained.

Table 10. *The changes in the epidermal mitotic activity of ten control mice and of ten mice injected with 3 mg. of phloridzin at 12.00 hr.*

Time of day	Average numbers of mitoses per unit section length	
	In control mice injected with saline	In mice injected with saline and phloridzin
10.00	2·8 ± 0·18	2·8 ± 0·23
11·00	1·8 ± 0·16	2·4 ± 0·21
12.00	2·9 ± 0·24	3·0 ± 0·20
13.00	4·2 ± 0·21	1·8 ± 0·17
14.00	7·5 ± 0·33	2·2 ± 0·29
15.00	6·2 ± 0·24	1·7 ± 0·18
16.00	3·1 ± 0·12	2·3 ± 0·20

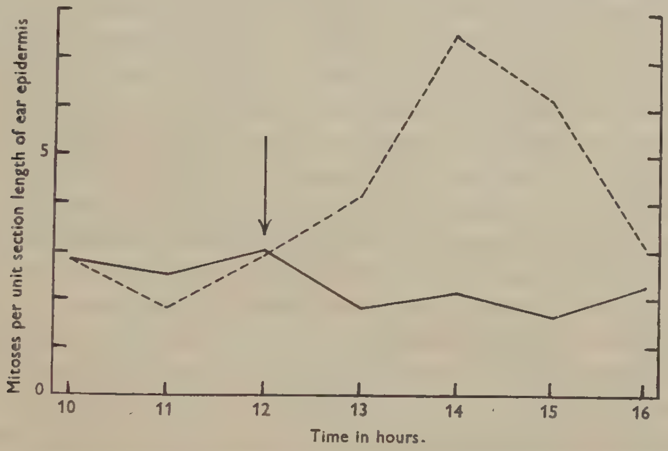

Fig. 9. The changes in the mitotic activity of the ear epidermis of mice injected at 12.00 hr. with 3 mg. of phloridzin (continuous line) as compared with controls (broken line).

IV. DISCUSSION

The results recorded above justify the general conclusion that a connexion exists between the concentration of the blood sugar and the mitotic activity of the ear epidermis. However, it is also apparent from these results, and from the observation that mitotic activity is normally high during sleep when the blood-sugar concentration is reduced, that the blood-sugar level *per se* is not the crucial factor which determines the form of the diurnal cycles. The actual control would appear to be exercised by some finer mechanism which is only crudely affected by unnaturally great changes in the blood-sugar concentration.

Before an attempt is made to discover the nature of this finer mechanism, it is perhaps important to try to arrive at some tentative conclusion regarding the role played by sugar in the process of cell division. Two obvious possibilities present themselves: either the glucose molecules are in some way built into the substance of the new cells, or they are destroyed to provide energy for the division. As to the first possibility, it is highly unlikely that the glucose molecule as such could be utilized as a building unit in the production of new cytoplasm. However, it is at least theoretically possible that it might be transformed into ribose, and then used in the formation of nucleoprotein. This possibility has been explored by means of subcutaneous injections of various concentrations of ribose dissolved in normal saline, but neither stimulation nor depression of the mitosis rate could be detected. It therefore appears improbable that in well-fed animals the supply of ribose can act as a limiting factor in cell division.

The second alternative, that glucose is of vital importance for the production of the energy consumed during cell division raises the question of whether, in fact, significant amounts of energy are involved in this process. The most recent work on this subject appears to be that of Zeuthen (1946, 1947) who, working first with frog's eggs and later with sea-urchin's eggs, concludes that there is an increase of about 10% in the respiration rate at or about the time of cleavage. This is in general agreement with the earlier results of Brachet (1934*a*, *b*), using frog's eggs, and of Runnström (1934), using sea-urchin's eggs. However, many other workers have entirely failed to distinguish any significant changes in the respiration rate during mitosis, and the question cannot be regarded as settled. Reviews of the extensive literature are given by Needham (1931, 1942) and Zeuthen (1946).

It must be concluded that the energy requirements of mitotic activity are at present unknown, and a suggestion that they may be significant can only be put forward in a tentative manner. However, it was partly because of this suggestion that experiments were carried out on the effects of injections of phosphate. It seemed possible that the relative deficiency of blood phosphate in the waking animal (Haldane, Wigglesworth & Woodrow, 1924) might act as a bar to the efficient phosphorylation of the sugar then available, while conversely its relative abundance in the sleeping animal might enable the development of full mitotic activity. This theory proved to be incorrect, but the results are of importance in indicating that injections of phosphate can augment the mitosis stimulating effect of injections of starch. This lends some support to the thesis that the function of glucose in cell division is to provide energy, and the depressing effects of phloridzin, which is known to interfere with the phosphorylation of sugar, also assume an added significance.

If sugar is as essential for cell division as would appear to be indicated, it is to be expected that its absorption by rapidly growing tissues of all kinds will prove to be high. That this is in fact so is well known. Many years ago Warburg described the extraordinary glycolytic powers of the cancer cell, and stated that in 10 hr. such a cell can destroy its own weight of sugar and convert it to lactic acid (Boyd, 1943). Similarly, the blood of the ungulate foetus has a higher sugar content than that of the mother, and it appears to be a general condition among mammals that the carbohydrate reserves of the embryo and placenta are not seriously reduced even when the greatest need for carbohydrates develops in the mother (Needham, 1942).

Relevant at this point is the other well-known fact that the vaginal epithelium becomes charged with glycogen when it develops high mitotic activity in response to oestrogenic hormones, and that coincidentally what is generally considered to be lactic acid appears in the vaginal lumen (Burrows, 1945). The establishment of such a local supply of carbohydrate would, on the present theory, be of the greatest assistance in the development of a high local mitosis rate. It is of interest to notice that, in apparent preparation for such a redistribution of glycogen, oestrogenic hormones cause a considerable increase in the blood-sugar level (Janes & Nelson, 1940). The detailed relation between glycogen deposition and mitotic activity in the cells of some tissue such as the vaginal epithelium is clearly an important subject for future investigation.

It is now possible to surmise why an unusual rise in the blood-sugar level does not infallibly induce an immediate increase in epidermal mitosis. It is clearly necessary that the sugar formed from the injected starch should be deposited into the epidermal cells, and this may not take place at once. In normal conditions, such deposition apparently occurs mainly during sleep, thus accounting simultaneously for the drop in the blood-sugar level and the rise in the mitosis rate. Relatively little appears to be known at the moment regarding the diurnal changes in the carbohydrate content of the epidermis and similar tissues. However, conditions in the liver are well known, and they confirm that deposition of sugar with the formation of glycogen is a characteristic of sleep (Brody, 1945). For the mouse liver, the most recent results are those of Dr P. J. Kipp (private communication), who estimated the variations in the glycogen content throughout a period of 24 hr. using an ingenious photoelectric method. The greater part of his results are reproduced in Fig. 10, in which, for comparison, the diurnal variations in the epidermal mitosis rate are also included. It is obvious that the times of glycogen deposition are approximately the same as the times of high mitotic activity, the slight lack of synchronization being probably explicable on the grounds of individual differences between the animals.

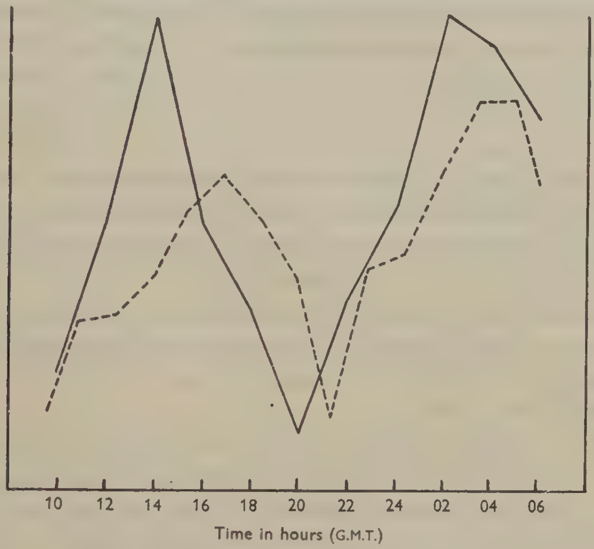

Fig. 10. The diurnal variations in the mitotic activity of the ear epidermis (continuous line) compared with the diurnal variations in the glycogen content of the liver (broken line). The liver glycogen graph is from results obtained by Dr P. J. Kipp.

It thus appears justifiable to adopt the working hypothesis that mitosis takes place most actively at those times when sugar is deposited from the blood into the tissues, and a full knowledge of the factors which initiate and control such deposition would seem to be important for a clear understanding of normal variations in the mitosis rate. Presumably one factor favouring deposition would be an excess of sugar in the blood stream, such as that caused in the present experiments by an injection of 20 mg. of starch. Another might be the temporary development of a greater area of contact between the tissue and the blood stream as induced by the dilation of blood vessels, by an increase in the numbers of capillaries, and by an increase in the rate of blood flow. These changes are seen, for instance, in the vicinity of wounds (Bullough, 1946), in the blood vessels of the skin after treatment with oestradiol (Reynolds, 1941), and around the hair bulbs in the region of rapid hair growth (Haddow, Elson, Roe, Rudall & Timmis, 1945). Yet another aid to mitosis stimulation might be an increase in the permeability of the capillary walls such as occurs in certain regions of the rat's body after treatment with oestrogens (Hechter, Krohn & Harris, 1942).

The opposite effect can be achieved by injections of insulin which, in a non-diabetic animal, are known to decrease the glycogen content of the liver as well as the blood-sugar level (Soskin & Levine, 1946). Local mitosis depression would also presumably follow a decrease in the blood supply, or a decrease in the permeability of the capillary walls.

If this theory is justified, it is evident that embryos, tumours, and such constantly proliferating tissues as lymph node centres must be characterized by an ability to absorb what sugar they require at all times of the day and night, even in conditions of unusual carbohydrate shortage.

In conclusion, it is interesting to consider the related question of the multiplication of blood parasites which has been investigated by Hegner (1937). He introduced the malarial parasite *Plasmodium cathemerium* into canaries made hyperglycaemic with glucose, or hypoglycaemic with insulin. In the former case the high blood-sugar level resulted in the production of an unusually large number of parasites, so that the birds sickened earlier and more frequently, and a greater proportion of them died. With insulin the opposite result was obtained with fewer parasites produced, and consequently with fewer illnesses and deaths among the hosts. Similar results have also been obtained by Steinbach & Duca (1942) with tuberculosis in rats. Animals which were rendered hyperglycaemic, and then inoculated intravenously with bovine tubercle bacilli, developed larger and more numerous tubercles than did the controls.

V. SUMMARY

1. An investigation has been made of the relation between the concentration of the blood sugar and the mitotic activity of the ear epidermis of the adult male mouse. It has been shown that, within limits, an increase in the blood-sugar level, induced by subcutaneous injections of starch, results in an increased mitosis rate. Conversely,

a decrease in the blood-sugar level, induced by insulin, causes a decreased mitosis rate. A deep mitosis depression is also caused by injections of phloridzin.

2. Since it is known that phloridzin interferes with the phosphorylation of sugar, and since it is also shown that injections of phosphate augment the effect produced by starch alone, the tentative suggestion is put forward that sugar may act by satisfying the energy requirements of mitosis.
3. However, in the normal animal the diurnal changes in the blood-sugar level are the inverse of the diurnal changes in mitotic activity, the concentration of the blood sugar being relatively low during sleep when the mitosis rate is relatively high. It is therefore evident that, within normal limits, the level of the blood sugar as such is not an important factor in the control of the diurnal mitosis cycles.
4. It is considered probable that the critical factor in the control of these cycles is the concentration of sugar, or glycogen, within the tissues themselves. Thus during sleep, sugar is probably deposited in the tissues, as it is known to be in the liver, so accounting simultaneously for the fall in the level of the blood sugar and the rise in the mitosis rate.

It is a pleasant duty to record my indebtedness to Dr and Mrs F. Bielschowsky and to Prof. H. A. Krebs, F.R.S., who were constantly ready to give advice of a biochemical nature, and to Dr A. Jordan who arranged for the blood-sugar estimations to be made in the laboratories of the Sheffield Royal Infirmary. The work was carried out during a tenure of the Sorby Research Fellowship of the Royal Society of London.

REFERENCES

Bissinger, E., Lesser, E. J. & Zipf, K. (1923). *Klin. Wschr.* **2**, 2233.
Boyd, W. (1943). *Text-book of Pathology*, 4th ed. London: Henry Kimpton.
Brachet, J. (1934*a*). *Arch. Biol., Paris*, **46**, 1.
Brachet, J. (1934*b*). *Arch. exp. Zellforsch.* **15**, 46.
Brody, S. (1945). *Bioenergetics and Growth.* New York.
Bullough, W. S. (1946). *Philos. Trans.* B, **231**, 453.
Bullough, W. S. (1948*a*). *Proc. Roy. Soc.* B, **135**, 212.
Bullough, W. S. (1948*b*). *Proc. Roy. Soc.* B, **135**, 233.
Bullough, W. S. (1949). *J. Exp. Biol.*, **26**, 76.
Burrows, H. (1945). *Biological Actions of the Sex Hormones.* Cambridge.
Haddow, A., Elson, L. A., Roe, E. M. F., Rudall, K. M. & Timmis, G. M. (1945). *Nature, Lond.*, **155**, 379.
Haldane, J. B. S., Wigglesworth, V. B. & Woodrow, C. E. (1924). *Proc. Roy. Soc.* B, **96**, 1.
Hechter, O., Krohn, L. & Harris, J. (1942). *Endocrinology*, **30**, 598.
Hegner, R. (1937). *J. Parasit.* **23**, 1.
Janes, R. G. & Nelson, W. O. (1940). *Proc. Soc. Exp. Biol., N.Y.*, **43**, 340.
Needham, J. (1931). *Chemical Embryology.* Cambridge.
Needham, J. (1942). *Biochemistry and Morphogenesis.* Cambridge.
Reynolds, S. R. M. (1941). *J. Invest. Dermat.* **4**, 7.
Runnström, J. (1934). *Protoplasma*, **20**, 1.
Simpson, G. G. & Roe, A. (1939). *Quantitative Zoology.* New York.
Soskin, S. & Levine, R. (1946). *Carbohydrate Metabolism.* Chicago.
Steinbach, M. M. & Duca, C. J. (1942). *Amer. Rev. Tuberc.* **46**, 304.
Zeuthen, E. (1946). *C.R. Lab. Carlsberg*, Ser. Chim., **25**, 191.
Zeuthen, E. (1947). *Nature, Lond.*, **160**, 577.

THE METABOLISM OF FERTILIZED AND UNFERTILIZED SEA-URCHIN EGGS

THE ACTION OF LIGHT AND CARBON MONOXIDE

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(With One Text-figure)

INTRODUCTION

The study of fertilization is still at a stage in which it is necessary to establish the existence of physiological differences between fertilized and unfertilized eggs. A number of differences have been found, and one of them has been considered particularly important. Runnström (1928, 1930) reported that unfertilized eggs were hardly affected by cyanide and carbon monoxide, though after fertilization these agents caused a marked inhibition of respiration. Runnström's experiments with cyanide seem to have been confirmed by Korr (1937), who concluded that the unfertilized eggs respire through a non-ferrous autoxidizable carrier, and that upon fertilization, functional connexion is established between dehydrogenase-substrates and the cyanide sensitive cytochrome oxidase by the 'throwing into circulation' (p. 483) of cytochrome.

Until now, cytochrome has not been observed spectroscopically in sea-urchin eggs (Brachet, 1934; Lindahl, 1936, 1939; Krahl, Keltch & Clowes, 1939; Ball & Meyerhof, 1940; Stotz, 1942). Ball & Meyerhof (1940), though failing to observe cytochrome in these eggs, reported that when pyridine was added to a reduced egg suspension, a typical haemochromogen spectrum was observed at 550–560 mμ. They suggested that sea-urchin eggs might therefore contain an iron-porphyrin catalyst other than cytochrome. Korr (1939) failed to observe the bands of cytochrome in these eggs, though the echinochrome, which it was thought might be responsible for the difficulties experienced in making spectroscopic examination, was made colourless by reduction with hyposulphite. Krahl, Keltch, Neubeck & Clowes (1941) showed that sea-urchin eggs contained as much cytochrome oxidase as many mammalian tissues, but reported that they contained no cytochrome *c*. Unfertilized eggs were found also to contain flavin adenine dinucleotide (Krahl, Keltch & Clowes, 1940), which led to the suggestion (Korr, 1942, p. 27) that respiration in the unfertilized egg is of a primitive type and passes directly through a flavoprotein to oxygen.

These hypotheses rest to a considerable extent on Runnström's and Korr's observations on the effects of inhibitors on the respiration of unfertilized eggs and it has become generally accepted in the literature that after fertilization oxidations are mediated through the cytochrome system, as shown by the inhibition of respiration by HCN and CO, 'in contrast with the insensitivity of the unfertilized eggs to these agents' (Goldinger & Barron, 1946, p. 73). On the other hand, Robbie (1946), in a careful series of experiments, found that 10^{-4} M-HCN almost completely inhibited the O_2 uptake of unfertilized eggs of *Arbacia punctulata*. A reinvestigation of the effect of CO on unfertilized and fertilized sea-urchin eggs (*Psammechinus miliaris*) was therefore of interest; it was combined with a spectroscopic examination of the eggs, which are often brilliantly coloured.

If, as Robbie found, low concentrations of cyanide reversibly inhibit the O_2 uptake of unfertilized eggs, their respiration is almost certainly mediated through the cytochrome system. In these circumstances, an explanation of the alleged failure to inhibit respiration with CO would be of some importance. Experiments on this subject are described in this paper.

The difficulties encountered in respirometric experiments on unfertilized sea-urchin eggs, because of their low O_2 consumption (approx. 28 μl. O_2/hr./10^6 eggs at 16° C.*), fragility and erratic behaviour, are probably responsible for the greater attention that fertilized eggs have received except in the work of Robbie (1946). For this reason and because comprehensive studies on the inhibition of respiration in fertilized eggs exist, unfertilized sea-urchin eggs have been extensively studied in this series of experiments.

MATERIAL

Jelly-free eggs of *P. miliaris* in sea water.

METHOD

Manometric procedure. O_2 consumption was measured in Barcroft-Warburg manometers, the vessels being conical and having a capacity of about 15 ml. 0·2 ml. of 10% KOH and filter-paper rolls were placed in the centre cups. Sperm or reagents were inserted in the side-arms. The vessels contained 3·0 ml. of egg suspension, which contained about 5.10^5 eggs. It was unnecessary to count the eggs, estimate the total nitrogen in the suspension, or measure the total volume of eggs in each experiment, as the control experiments were done on the experimental suspensions. The reasons for doing the experiments in this way are given later. The temperature was 16·0° C. and the shaker rate 80 c.p.m. with a 5 cm. stroke. O_2 saturation was satisfactory in these conditions. At the end of a 3 hr. experimental period, the eggs had not cytolysed and were more than 90% fertilizable. In cases where cytoplasmic debris was observed in the sea water round the eggs, the results were discarded. According to Runnström (1930), the O_2 uptake of unfertilized eggs increases after prolonged agitation in the manometers and the fraction of the respiration corresponding to this increase is CO-sensitive. This increase in O_2 uptake has not been observed in the unfertilized eggs of *P. miliaris* at 16° C. during short periods in the manometers, but after 90 min. it occurred in some batches of eggs. The eggs, however, were still fertilizable. Robbie (1946) apparently did not observe this phenomenon in the eggs of *Arbacia punctulata*.

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* Using the data of Harvey (1931) and McClendon (1909), 10^6 eggs ≒ 0·32 ml. egg substance ≒ 0·35 g. wet weight ≒ 0·06 g. dry weight. The Q_{O_2} is therefore about 0·5.

Gas mixtures. Cylinder CO, O_2 and N_2 mixtures were used. To save space, the proportions of these gases used in an experiment are indicated by putting the percentage of CO or N_2 in the index position. CO^{95}/O_2 means 95 % CO, 5 % O_2, and $CO^{70}N_2^{10}/O_2$ means 70 % CO, 10 % N_2, 20 % O_2. The cylinder CO was tested on sea-urchin spermatozoa and gave the same results as CO prepared by mixing formic and sulphuric acid. After gassing, the vessels were equilibrated for 10 min. in the bath before zero readings were taken.

Controls. Runnström (1930) made duplicate measurements, one vessel containing eggs and CO/O_2 mixtures and the other eggs with air in the gas space. He said it was unnecessary to have the conventional N_2/O_2 mixtures in the control vessels, as the O_2 uptake of the eggs was unaffected by reducing the O_2 tension from 153 mm. Hg (air) to 38 mm. Hg (the control appropriate for an experiment in which the gas space contains CO^{95}/O_2). This observation is confirmed at a shaker speed of 80 c.p.m. There is, however, a possible objection to this method of running controls, which was also used by Lindahl (1939). Different batches of eggs, in different manometers, do not always respire at the same rate over extended periods. It was therefore thought safer to use the same eggs for controls and CO experiments provided that the period of measurement was not sufficiently long to provoke the increase in O_2 uptake to which reference has already been made. The O_2 consumption was first measured in the presence of N_2/O_2. The CO/O_2 mixture was then introduced and further measurements made. The reverse procedure, starting with CO/O_2 and replacing this with N_2/O_2, produces misleading results which are discussed later.

Illumination. 750 W. carbon arc shining on to the bottoms of the manometer vessels. During dark periods, the vessels were placed in lined black bags.

Spectroscopic examination. Microscope spectroscope; eggs brought to temperature of liquid air (Keilin & Hartree, 1939).

RESULTS

Influence of light on unfertilized eggs. This was examined because of a peculiar result obtained when unfertilized and fertilized eggs were illuminated in the presence of CO (Fig. 1). In this experiment, the O_2 uptake of the unfertilized eggs was measured for 30 min. in air. CO^{80}/O_2 was then introduced and a further measurement was made for 30 min. in the dark. The vessel was illuminated during the next half-hour period and after this sperm were tipped into the egg suspension. Fertilization was successful, as evidenced by the increase in O_2 uptake after insemination. This was confirmed at the end of the experiment. After the respiration of the fertilized eggs had been measured in CO^{80}/O_2 in the light, a further measurement was made in the dark. Fig. 1 shows that the O_2 uptake of unfertilized eggs is stimulated by CO^{80}/O_2 in the dark, but that light has a small inhibitory action. There is the usual increase in respiration on fertilization, after which CO^{80}/O_2 has its characteristic photo-reversible inhibitory effect. It will be shown that these

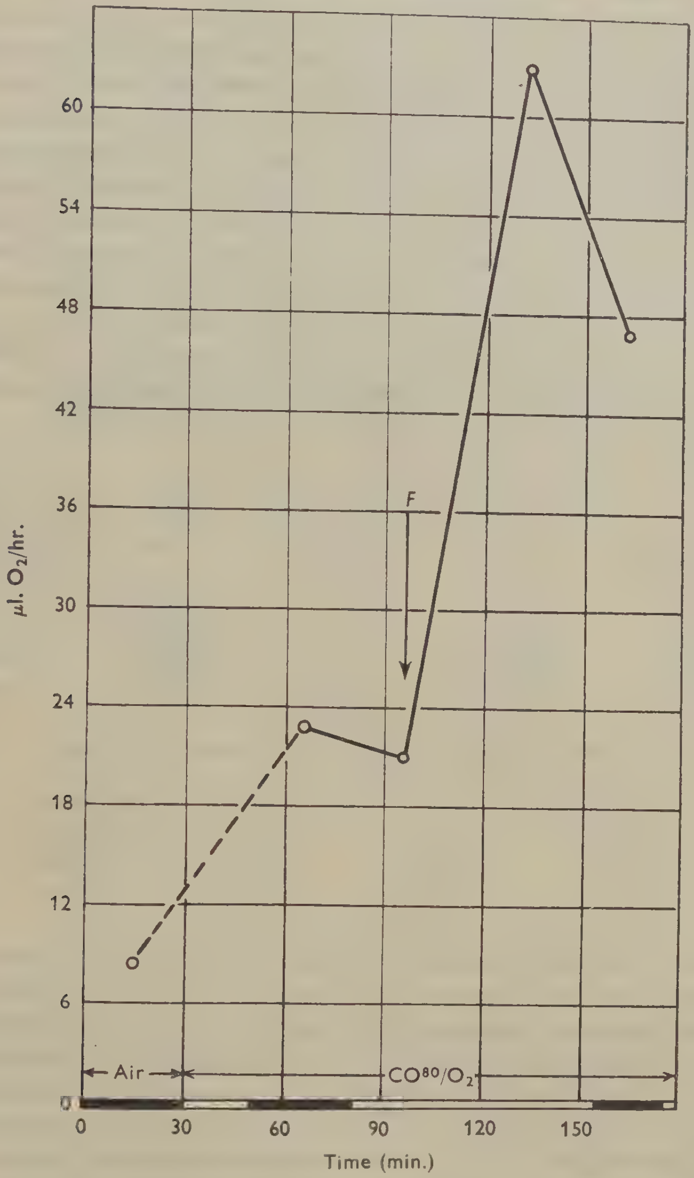

Fig. 1. Effect of 80 % CO in O_2 on O_2 uptake of unfertilized and fertilized eggs of *Psammechinus miliaris* in light and darkness. Fertilization, *F*, *in situ*. The black and white blocks under the base-line refer to alternating periods of darkness and illumination by carbon arc. The points at $t=15$, 65, 132·5 and 162·5 min. are the means of 30, 30, 30, 45 and 25 min. readings. In the interval $t=30$–50, CO^{80}/O_2 was substituted for air above the eggs. The graph shows the increase in O_2 uptake in CO^{80}/O_2 in the dark, and decrease in light; the increase in O_2 uptake after fertilization in CO^{80}/O_2 in light, and the inhibitory action of CO^{80}/O_2 on fertilized eggs in darkness. *T*° C., 20.

changes in metabolism have the following causes: increase in O_2 uptake in the presence of CO;* inhibition of O_2 uptake by CO; inhibition of O_2 uptake by light.

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* The increase in O_2 uptake in the presence of CO will be called 'CO-respiration'.

The average inhibition of respiration in light and air was 38% (forty-four experiments). The most convincing way of proving that light inhibits respiration is by measuring the O_2 uptake during four consecutive half-hour periods, two in darkness and two in light. Table 1 is an example of such an experiment done in duplicate. This way of demonstrating the effect of some treatment on unfertilized eggs has the disadvantage that it necessitates a rather long period of agitation in the manometers, which causes an increase in O_2 uptake. Nevertheless, whichever dark and light periods are compared, respiration is inhibited by light. Although the increase in respiration with time does not obscure the interpretation of the experiment in this case, it may do so in others, especially in studies with CO. So far as possible, experiments were therefore arranged to last for short periods, i.e. 60 min., the longer ones only being done when some particular point required special verification. These difficulties do not arise in experiments with fertilized eggs.

Table 1. *Unfertilized* Psammechinus miliaris *eggs. Gas phase, air*

Time (min.)	µl. O_2/hr.	Increase in dark (%)	µl. O_2/hr.	Increase in dark (%)
0–30	18, dark	39	17, light	47
30–60	14, light	64	25, dark	14
60–90	23, dark	21	22, light	32
90–120	19, light		29, dark	

Table 2. *Unfertilized* Psammechinus miliaris *eggs*

Time (min.)	Gas	µl. O_2/hr.	µl. O_2/hr.
0–30	CO^{95}/O_2	9, dark	11, dark
30–60	CO^{95}/O_2	21, light	21, light
0–30	N_2^{95}/O_2	20, dark	20, dark

O_2 *uptake of unfertilized eggs in the presence of* CO^{95}/O_2. In this series, the respiration of unfertilized eggs was first measured in N_2^{95}/O_2, and then in CO^{95}/O_2. The reverse procedure, measuring the O_2 uptake in the presence of CO first, and then in the N_2/O_2 gas mixture, gives results which are liable to misinterpretation. An example is shown in Table 2. The eggs were equilibrated with CO^{95}/O_2 and their O_2 uptake measured in light and darkness. The manometer vessels were then thoroughly gassed with N_2^{95}/O_2 and a further measurement made in the dark. Superficially, it seems that O_2 uptake in CO^{95}/O_2 in the dark is half that in N_2^{95}/O_2, and illumination of the CO-inhibited suspension causes complete photo-reversal. Experiments in which O_2 uptake is first determined in N_2^{95}/O_2 and subsequently in CO^{95}/O_2 (Table 3) show without doubt that this is a false interpretation. The high O_2 uptake of eggs in nitrogen is caused by the stimulating effect of the previous CO treatment, sufficient CO remaining after gassing with N_2/O_2 to produce the observed increase in respiration. Table 3 appears to show that there is negligible inhibition of respiration by CO in the dark; on the other hand, there is a marked increase in O_2 uptake when the eggs are illuminated in CO.

Fourteen comparisons were made of the O_2 uptake of unfertilized eggs in N_2^{95}/O_2 and CO^{95}/O_2 in the dark. In three of these there was a decrease in respiration in CO^{95}/O_2, av. 11 %; in two there was no difference; while in the remaining nine, there was an average increase in CO^{95}/O_2 of 14 %. Twenty-four determinations of the respiration in CO^{95}/O_2 in dark and light were made. The average increase in light was 44 %. This figure is important for comparison with the effect of light at lower partial pressures of CO.

O_2 *uptake of unfertilized eggs in the presence of* CO^{80}/O_2. Interpretation of the results in the previous section requires experiments either at higher or lower CO partial pressures. The former, with CO^{97}/O_2 for example, are not satisfactory, as this concentration of CO is often harmful to unfertilized eggs. A similar irreversible toxic action of high partial pressures of CO has been observed in sea-urchin spermatozoa (Rothschild, 1949). Fig. 1 shows that with a gas mixture of CO^{80}/O_2 there is a slight decrease in O_2 uptake when the eggs are illuminated. In CO^{95}/O_2, however, it will be remembered that illumination causes an increase of 44 %. CO^{80}/O_2 contains a critical partial pressure of CO, at which photo-reversal of inhibition and light inhibition approximately balance each other, one or other of these two factors predominating according to the susceptibilities of the eggs from different batches. Sixteen experiments, one of which is shown in Table 4, were done to compare the O_2 uptake in CO^{80}/O_2 in the dark and in N_2^{80}/O_2. The average increase in respiration in CO^{80}/O_2, as compared with N_2^{80}/O_2, was 55 %. This figure should be compared with the analogous one for CO^{95}/O_2, 7 %. On the other hand, as shown in Fig. 1, illumination causes no increase in O_2 uptake with CO^{80}/O_2, though in CO^{95}/O_2 the increase was 44 %. Nine experiments to verify this result are recorded in Table 5.

Table 3

Time (min.)	Gas	µl. O_2/hr.	µl. O_2/hr.	µl. O_2/hr.
0–30	N_2^{95}/O_2	15, dark	20, dark	15, dark
30–60	N_2^{95}/O_2	13, light		
0–30	CO^{95}/O_2	14, dark	25, light	17, dark
30–60	CO^{95}/O_2	20, light	19, dark	24, light
60–90	CO^{95}/O_2	15, dark	27, light	17, dark
90–120	CO^{95}/O_2	21, light	22, dark	25, light

Table 4. *Unfertilized* Psammechinus miliaris *eggs*

Time (min.)	Gas	µl. O_2/hr.	µl. O_2/hr.
0–30	N_2^{80}/O_2	24, dark	23, dark
0–30	CO^{80}/O_2	35, dark	33, dark

Table 5 shows that there is no increase in O_2 uptake in CO^{80}/O_2 in light, illumination having a small inhibitory effect in general. As mentioned earlier, this partial pressure of CO is such that light inhibition of O_2 uptake and photo-reversal of inhibition approximately balance each other; the susceptibility to CO and light of any particular batch of eggs determines which of the two effects will predominate. In these circumstances variable or apparently inconsistent results are to be expected, and in fact occur.

O_2 *uptake of unfertilized eggs in lower partial pressures of* CO. A few experiments were done at $CO^{60}N_2^{20}/O_2$ (5), $CO^{70}N_2^{10}/O_2$ (3), and $CO^{50}N_2^{30}/O_2$ (1). The light inhibition was pronounced in these cases, but the increase in respiration in CO did not differ significantly from that in CO^{80}/O_2.

Influence of light on fertilized eggs. The O_2 uptake of fertilized eggs is slightly inhibited by light, the average inhibition in fifty-three experiments being 9%. Although the effect is small, and nearly within the limits of experimental error, there is no doubt of its existence in certain batches of fertilized eggs, see Table 6.

Table 5. *Unfertilized* Psammechinus miliaris *eggs in* CO^{80}/O_2. O_2 *uptake for 30 min., in* $\mu l.\ O_2/hr.$

Dark	18	15	23	24	35	33	24	23	31
Light	17	15	21	23	23	24	30	25	28

Table 6. *Inhibition of* O_2 *uptake by light in fertilized* Psammechinus miliaris *eggs. Gas phase, air*

Time (min.)	$\mu l.\ O_2/hr.$	$\mu l.\ O_2/hr.$
0–15	69, light	52, dark
15–30	77, dark	50, light
30–45	73, light	52, dark
45–60	83, dark	49, light

As mentioned in the Introduction, few investigations were made on the effect of CO on the respiration of fertilized eggs, as this field has been comprehensively covered by Runnström (1930) and Lindahl (1939). The stimulating action of CO, observed by Lindahl, was confirmed.

Cytochrome in unfertilized eggs. Cytochromes *a* and b_1 (cf. Keilin & Harpley, 1941) were spectroscopically identified in unfertilized eggs.

DISCUSSION

In this section, the following interpretation of the data in the Results section will be developed:

(1) The O_2 uptake of unfertilized sea-urchin eggs is catalysed by the cytochrome system and is photo-reversibly inhibited by CO.

(2) We should therefore expect the O_2 uptake in CO in the dark to be lower than the O_2 uptake in air or the appropriate control gas mixture. It is not, and this is due to CO increasing and inhibiting respiration at the same time.

(3) If it is true that CO stimulates and inhibits respiration at the same time, we should be able to separate these two effects by making use of the fact that CO-

cytochrome oxidase is dissociated by light. In this case, O_2 uptake in CO in light should be greater than in the dark, and, as the inhibition of O_2 uptake is proportional to the ratio $(CO)/(O_2)$, the difference between O_2 uptake in CO in dark and light will be greater at high partial pressures of CO than at low ones. This has been found to be the case.

(4) Interpretation is made somewhat complicated at low partial pressures of CO, by the inhibitory action of light on O_2 uptake, in the presence or absence of CO.

At this stage it is convenient to discuss the researches of Lindahl (1939) on the reaction of sea-urchin eggs to CO; as his results differ in several particulars from those described here, and in some cases when the results are in agreement his interpretation is not the same, an examination of the controversial points is made below. At the same time (1)–(4) will be amplified.

Cytochrome in eggs. Lindahl casts doubt on the existence of cytochrome in eggs and, incidentally, in spermatozoa, of sea-urchins. In the case of the latter, cytochrome has been observed spectroscopically by Ball & Meyerhof (1940) and Rothschild (1948). In the case of eggs, Krahl *et al.* (1941) identified cytochrome oxidase chemically, while the spectroscopic identification of cytochromes a and b_1 in sea-urchin eggs is reported in this paper. Spectroscopic identification of a_3 is prevented by the egg pigments.

Effect of CO *on unfertilized eggs.* According to Lindahl (1939, p. 152), CO does not inhibit respiration. He found, however, that O_2 uptake in CO and light was greater than in CO and darkness at all partial pressures of CO. I have been unable to confirm this result, for in the experiments recorded above, light, so far from stimulating respiration even at fairly high CO partial pressures (CO^{80}/O_2), has an inhibitory action. The presence of CO in the gas phase has no bearing on this light inhibition, as it occurs in the absence of CO. Lindahl states that the increased O_2 uptake in CO and light could be due to photo-reversal of CO-inhibited respiration. He points out that if this were so, the ratio (Increased O_2 uptake in the dark/Increased O_2 uptake in the light) should be inversely proportional to the partial pressure of CO; in his experiments, the ratio remained constant at different CO partial pressures. I have also been unable to confirm this result: for it will be remembered that the difference between O_2 uptake in CO^{80}/O_2 in the dark and in light was insignificant, while in CO^{95}/O_2, illumination caused an increase of 44%. This proves that respiration is mediated through the cytochrome system, though the experiments do not distinguish between cytochrome catalysis of normal and CO-respiration. If further proof were needed, apart from the existence of cytochromes a, b_1, and cytochrome oxidase, O_2 uptake in CO^{95}/O_2 and CO^{80}/O_2 can be compared with their appropriate controls. The former caused an increase in O_2 uptake of about 14%, while the latter increased respiration by 55%. The higher partial pressure of CO clearly inhibits respiration, and this is reversed by light. Because of the simultaneous stimulating action of CO, the inhibition is evidenced by there being a smaller stimulating action at high partial pressures of CO than at low ones. As Runnström observed, the respiration of the controls was the same, whether the gas mixture was N_2^{95}/O_2 or N_2^{80}/O_2; the results are therefore not due to inadequate oxygen saturation.

Lindahl suggests that some pigment of an unknown type is responsible for the *increased* O_2 uptake in CO and light. In *P. miliaris* eggs, however, light *decreases* respiration, the unfertilized eggs being more affected than fertilized ones. The yellow or orange pigment which colours the eggs could be involved in this inhibition, but no information is available. The observation of Hartmann & Schartau (1939) that light inhibits the activating effect of echinochrome A on the spermatozoa of *Arbacia postulosa* is of interest in this connexion.

Lindahl (1939), on the basis of his experiments, concluded that the 'O_2-überführende Ferment' was not concerned in the respiration of unfertilized eggs (p. 161), and that the slight inhibition with KCN observed by Runnström (1930) was caused by the combination of KCN with 'indophenol oxidase' alleged to be unaffected by CO. This concept is based on an oxidation scheme due to Tamiya & Ogura (1937), which, according to Lindahl, explains his results on sea-urchin eggs:

$$\mathrm{O_2} - \text{‘}\overset{\substack{\mathrm{CO}\\ \downarrow}}{\mathrm{O_2}\text{-überführende Ferment}}\text{’} - \text{indophenol } \overset{\substack{\mathrm{CN}\\ \downarrow}}{\text{oxidase}} - \text{cytochromes } c,\ a,\ b.$$

As the 'oxygen-transferring enzyme' and indophenol oxidase are identical, and now known as cytochrome oxidase (or a_3), and as both CN and CO inhibit cytochrome oxidase,* the introduction of two oxygen-transferring enzymes into this scheme of oxidation is superfluous.

The experiments described in this paper show that the cytochrome system is present and functional in unfertilized sea-urchin eggs. Runnström (1930) came to the same conclusion from studying the increased O_2 uptake of unfertilized eggs in the presence of dimethyl *p*-phenylene diamine (Dpphd). He interpreted the apparent insensitivity of the eggs to cyanide and CO as indicating a lack of contact between cytochrome and substrate (1930), or to a block in the chain of carriers (1935). Robbie (1946) showed that at the beginning of his manometric experiments, 10^{-4} M-HCN almost completely inhibited the O_2 uptake of unfertilized sea-urchin eggs; he attributed previous failures to observe this inhibition to inadequate control of HCN concentration, which it is well known requires special precautions in manometric experiments involving the absorption of CO_2 by KOH. The absence of extensive measurements on the effect of light on eggs in CO may be the explanation of previous failures to observe CO inhibition of unfertilized egg respiration, though this does not apply in the case of Lindahl's results and interpretations which have already been discussed.

There is one question which has not been resolved. Does CO inhibit the normal respiration of the unfertilized egg, or the CO-respiration? Runnström (1930) showed that the increased respiration in the presence of Dpphd was strongly inhibited by CO and it may be that CO in light, like Dpphd, acts as a substrate which contributes towards the saturation of the cytochrome system, inhibition in the dark occurring at the other end of the metabolic sequence. If this is so, CO-inhibition of CO-respiration can only be partial, for if it were complete, there could obviously be no increased O_2 uptake in the presence of CO. On the other hand, Robbie's HCN experiments provide a good case for the view that CO does inhibit the normal O_2 uptake of the eggs. This point might be clarified by examining the inhibitory action of HCN on unfertilized eggs in the dark, in the presence of CO, but at a sufficiently low partial pressure to avoid CO inhibition of cytochrome oxidase.

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* CN inhibits the reduction of ferri-a_3, and CO the oxidation of ferro-a_3.

There is a superficial resemblance between the unfertilized sea-urchin egg and *B. coli*: first, Krahl *et al.* (1941) were unable to find any cytochrome *c* in the former, while Keilin & Harpley (1941) showed that *B. coli* are devoid of this carrier. Secondly, neither organism has the *b* and *c* bands of a typical cytochrome; instead there is the single band b_1 which, in the case of *B. coli*, represents the α-band of a non-autoxidizable haemochromogen. The resemblance stops at this point, because *B. coli* does not contain cytochrome oxidase while the sea-urchin egg does. Furthermore, in the former, the inhibition of respiration is not light sensitive. Yamagutchi (1937) asserted that *B. coli* contained cytochrome *c*, its band being masked by b_1. Keilin & Harpley (1941) have shown that this is not correct, but in view of the unfertilized sea-urchin egg's ability to oxidize Dpphd, this interpretation of the spectrum might apply to the eggs, even though it is wrong in the case of *B. coli*. Further experiments, similar to those of Keilin & Harpley on *B. coli*, are required to clarify this point.

Although these problems may be of intrinsic interest, they are unlikely to throw any light on the nature of the stimulus to development caused by adhesion of a spermatozoon to the egg surface. The increased O_2 uptake of sea-urchin eggs after fertilization is not observed in other eggs and is therefore unlikely to have any deep significance in the fertilization reaction. The mere fact that so many diverse treatments, such as prolonged agitation, an increase in temperature (Korr, 1937; Laser & Rothschild, 1939), pyocyanine (Runnström, 1935) or Dpphd (Runnström, 1930), can cause increases in the O_2 uptake of unfertilized eggs without parthenogenetic activation, and in some cases without loss of fertilizability, is sufficient to show that the different respiratory levels of fertilized and unfertilized eggs are of secondary importance. The difference in the sensitivity of unfertilized and fertilized sea-urchin eggs to inhibitors of cytochrome oxidase, a difference which is not in dispute, is probably correlated with the difference in respiratory activity before and after fertilization,* but the concept that after fertilization cytochrome is 'thrown into circulation' must be rejected, for the simple reason that cytochrome is 'in circulation' before fertilization has occurred. Runnström, who did not subscribe to the cytochrome release theory, has discussed in detail the possible explanations of the different respiratory rates of sea-urchin eggs before and after fertilization. Ballentine (1940) and Brachet (1947, pp. 132–6) have also examined this subject.

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* Warburg (1927) pointed out that unless a cell is rapidly oxidizing substrate, it cannot be expected to show great inhibition of O_2 uptake in the presence of CO.

Neither the inhibitory action of light on unfertilized eggs, nor the much reduced effect on fertilized eggs, have been noted before. As is so often the case in the study of fertilization, the importance of this observation can only be assessed when other eggs have been examined, to see if they react in the same way.

The most probable explanation of the stimulating action of CO is that it is oxidized, ultimately, to CO_2. The reaction is energetically probable and has been shown to occur in other tissues such as heart muscle (Fenn & Cobb, 1932*a*, *b*) and *Actinomyces oligocarbophilus* (Lantsch, 1922). Fenn & Cobb (1932*a*, p. 386) state that the increased oxidation in the presence of CO 'seems to be mediated by the normal oxidizing system'. In confirmation of this view, KCN was found to inhibit the CO-respiration. They concluded (p. 390) that 'the stimulating effect of CO seems to be in reality a phenomenon which is superimposed upon an inhibition due to CO', the same conclusion that has been reached from the experiments described in this paper. Fenn & Cobb did not observe any respiratory increase when the tissue was illuminated in the presence of CO. This was clearly due to the light source being too weak, and subsequently, Schmitt & Scott (1934) found that illumination of skeletal and cardiac muscle in CO did cause an increase in O_2 uptake, as it does in unfertilized sea-urchin eggs when the partial pressure of CO is high enough to cause a significant inhibition.

Fenn & Cobb's experiments on the inhibition of CO-respiration by KCN indicate that the cytochrome system is involved in the oxidation of CO. It is suggestive in this connexion that Negelein (1931) found that CO is oxidized to CO_2 by hemin in M-NaOH, if the O_2 tension is sufficiently low.

SUMMARY

1. An investigation has been made into the effect of light and carbon monoxide on the respiration of sea-urchin eggs (*Psammechinus miliaris*), with special reference to unfertilized eggs.

2. The O_2 uptake of unfertilized eggs in air is 38% higher in the dark than in light. This inhibitory action of light persists when gas mixtures containing CO and O_2 are substituted for air.

3. Fertilized and unfertilized eggs differ in that the former's respiration is only about 10% inhibited by light.

4. In 80% CO in O_2 in the dark, the O_2 uptake of unfertilized eggs is 55% higher than in 80% N_2 in O_2 in the dark.

In 95% CO in O_2 in the dark, the O_2 uptake of unfertilized eggs is only 7% higher than in 95% N_2 in O_2 in the dark, showing that at this partial pressure, CO exerts an inhibitory action on respiration.

5. There is no difference between O_2 uptake in 80% CO in O_2 in light and dark.

O_2 uptake in 95% CO in O_2 in light is 45% higher than in this gas mixture in the dark, showing that at this partial pressure of CO, photo-reversal of respiratory inhibition occurs.

6. Results similar to those in 80% CO in O_2 were obtained with gas mixtures containing lower partial pressures of CO.

7. Cytochromes a and b_1 were identified spectroscopically in unfertilized eggs.
8. It is concluded that respiration is mediated through the cytochrome system before and after fertilization. In theories which postulate that cytochrome is 'thrown into circulation' at fertilization, the simultaneous light-inhibition of respiration, stimulation of O_2 uptake by CO, and photo-reversible inhibition of O_2 uptake by CO have not been taken into consideration.

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REFERENCES

Ball, E. G. & Meyerhof, B. (1940). *J. Biol. Chem.* **134**, 483.
Ballentine, R. (1940). *J. Cell. Comp. Physiol.* **15**, 217.
Brachet, J. (1934). *Arch. Biol.* **45**, 611.
Brachet, J. (1947). *Embryologie Chimique.* Paris: Masson and Cie.
Fenn, W. O. & Cobb, D. M. (1932*a*). *Amer. J. Physiol.* **102**, 379.
Fenn, W. O. & Cobb, D. M. (1932*b*). *Amer. J. Physiol.* **102**, 393.
Goldinger, J. M. & Barron, E. S. G. (1946). *J. Gen. Physiol.* **30**, 73.
Hartmann, M. & Schartau, O. (1939). *Biol. Zbl.* **59**, 571.
Harvey, E. N. (1931). *Biol. Bull. Woods Hole*, **61**, 273.
Keilin, D. & Hartree, E. F. (1939). *Proc. Roy. Soc.* B, **127**, 167.
Keilin, D. & Harpley, C. H. (1941). *Biochem. J.* **35**, 688.
Korr, I. M. (1937). *J. Cell. Comp. Physiol.* **10**, 461.
Korr, I. M. (1939). *Cold Spr. Harb. Symp. Quant. Biol.* **7**, 418.
Korr, I. M. (1942). *A Symposium on Respiratory Enzymes.* University of Wisconsin Press.
Krahl, M. E., Keltch, A. K. & Clowes, G. H. A. (1939). *Biol. Bull. Woods Hole*, **77**, 318.
Krahl, M. E., Keltch, A. K. & Clowes, G. H. A. (1940). *Proc. Soc. Exp. Biol., N.Y.*, **45**, 719.
Krahl, M. E., Keltch, A. K., Neubeck, C. E. & Clowes, G. H. A. (1941). *J. Gen. Physiol.* **24**, 597.
Lantsch, K. (1922). *Zbl. Bakt.* (2. Abt.), **57**, 309.
Laser, H. & Rothschild, Lord (1939). *Proc. Roy. Soc.* B, **126**, 539.
Lindahl, P. E. (1936). *Acta zool., Stockh.*, **17**, 179.
Lindahl, P. E. (1939). *Z. vergl. Physiol.* **27**, 137.
McClendon, J. F. (1909). *Amer. J. Physiol.* **23**, 460.
Negelein, E. (1931). *Biochem. Z.* **243**, 386.
Robbie, W. A. (1946). *J. Cell. Comp. Physiol.* **28**, 305.
Rothschild, Lord (1948). *J. Exp. Biol.* **25**, 15.
Rothschild, Lord (1949). *J. Exp. Biol.* **25**, 353.
Runnström, J. (1928). *Acta zool., Stockh.*, **9**, 445.
Runnström, J. (1930). *Protoplasma*, **10**, 106.
Runnström, J. (1935). *Biol. Bull. Woods Hole*, **68**, 327.
Schmitt, F. O. & Scott, M. G. (1934). *Amer. J. Physiol.* **107**, 85.
Stotz, E. (1942). *Cytochromes. A Symposium on Respiratory Enzymes.* University of Wisconsin Press.
Tamiya, H. & Ogura, Y. (1937). *Act. phytochim., Tokyo*, **9**, 123.
Warburg, O. (1927). *Biochem. Z.* **189**, 354.
Yamagutchi, O. (1937). *Bot. Mag., Tokyo*, **51**, 457.